Modulatory Potential of Fuzapladib and Pentosan Polysulfate Sodium on Adhesion Molecules and Chemokine Receptors in canine endothelial cells

(犬血管内皮細胞におけるケモカイン受容体機能と接着因子発現に対するフザプラジブおよびポリ硫酸ペントサンの干渉能)

Darawiroj Kanittha

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Table of Contents

Abbreviations

%	Percent
$2^{-\Delta\Delta Ct}$	Two to the power of minus delta delta cycle threshold
7-AAD	7-Aminoactinomycin D
AFS	Attachment Factor Solution
ANOVA	Analysis of Variance
Calcein-AM	Calcein acetoxymethyl ester
Cat. No.	Catalog number
CD11a	Cluster of Differentiation 11a
CD18	Cluster of Differentiation 18
CINC	Cytokine-induced neutrophil chemoattractant
CnAOEC	Canine aortic endothelial cells
CXCL1	C-X-C motif chemokine ligand 1
CXCR2	C-X-C motif chemokine receptor 2
DMOAD	Disease-modifying osteoarthritis drug
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FI	Fluorescence intensity
FZP	Fuzapladib sodium hydrate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HBSS	Hanks' Balanced Salt Solution
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
K_2EDTA	Dipotassium ethylenediaminetetraacetic acid
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
MEXT	Ministry of Education, Culture, Sport, Science and Technology
MFI	Mean fluorescence intensity
ml	Milliliter
mRNA	Messenger ribonucleic acid
ng	Nanogram

PBS	Phosphate-buffered saline
PE	Phycoerythrin
pg	Picogram
PPS	Pentosan polysulfate sodium
PSGL-1	P-selectin glycoprotein ligand-1
qPCR	Quantitative real-time polymerase chain reaction
rh-cTNFα	Recombinant canine tumor necrosis factor alpha
RM	Repeated-measures
RM one-way ANOVA	Repeated-measures one-way analysis of variance
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute 1640 medium
SD	Standard deviation
µg	Microgram
µl	Microliter
µM	Micromolar

Notes

Chapter I: Darawiroj K, Sunaga T, Owaki R, Handondo NM, Okumura M. Effect of Fuzapladib Sodium Hydrate on Adhesion Molecule Expression in Canine Endothelial Cells and Neutrophils. J Vet Med Sci Advance online publication, 2025. doi:10.1292/jvms.25-0507.

Chapter II: Darawiroj K, Sunaga T, Owaki R, Handondo NM, Okumura M. Effect of Pentosan Polysulfate Sodium on Adhesion Molecule Expression in Canine Endothelial Cells and Neutrophils. Jpn J Vet Res In press.

Preface

Inflammation is a response that allows the body to identify and eliminate harmful stimuli such as pathogens, damaged cells, irritants, or toxic substances[8, 38]. It begins with coordinated vascular and cellular events. These include increased vascular permeability, the release of pro-inflammatory mediators, and the rapid recruitment of neutrophils to inflamed tissues. Together, these processes lead to the classical signs of redness, swelling, heat, pain, and functional impairment[8, 51]. Inflammation is commonly divided into acute and chronic phases[16]. During the acute phase, neutrophils are rapidly recruited to the inflamed tissue through a tightly regulated sequence of interactions between adhesion molecules expressed on both neutrophils and endothelial cells. These interactions mediated by selectins, integrins, and members of the immunoglobulin superfamily, coordinate neutrophil tethering, rolling, firm adhesion, and transendothelial cell migration[14]. In addition to eliminating pathogens, neutrophils also release cytokines that affect other immune cells and contribute to the resolution of inflammation[51].

Depending on the severity and persistence of the stimulus, the acute response may be sufficient to restore tissue homeostasis, or it may progress to a chronic state characterized by mononuclear cell infiltration, progressive tissue injury, and fibrosis[7]. Chronic inflammation has been associated with a wide range of diseases, including arthritis, asthma, atherosclerosis, autoimmune disorders, diabetes, cancer, and age-related degenerative conditions[16]. Moreover, systemic inflammation can result in acute complications such as lung and renal injury, encephalomyelitis, colitis, and endotoxemia[19]. Excessive or uncontrolled inflammation may lead to physiological disturbances, tissue destruction, organ dysfunction, or even death[56]. Given the central role of neutrophils in initiating and amplifying these responses, a clear understanding of the neutrophil recruitment cascade is essential.

Neutrophil recruitment and transmigration are regulated by a tightly coordinated multistep cascade that directs circulating neutrophils from the bloodstream to sites of inflammation[37], as schematically illustrated in Figure 1. This sequence begins with selectin-mediated tethering and rolling on activated endothelial cells. Neutrophils express P-selectin glycoprotein ligand-1 (PSGL-1), which binds endothelial E-selectin and P-selectin to establish the first adhesive contact[33, 37, 73]. Rolling is further supported by neutrophil L-selectin, which facilitates secondary capture through interacting with PSGL-1 on adjacent rolling neutrophils. In turn, this promotes slow and sustained transit along the endothelium[40, 61]. Selectin-PSGL1 interaction transduces an intracellular signal

that partially activates lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18). This shifts the integrin from a low-affinity state to an intermediate-affinity state. Consequently, LFA-1 is enabled to bind intercellular adhesion molecule-1 (ICAM-1) on endothelial cells and support slow rolling[32].

During rolling, endothelial cells secrete the chemokine C-X-C motif chemokine ligand 1 (CXCL1), which binds to C-X-C motif chemokine receptor 2 (CXCR2) on neutrophils, triggering a rapid inside-out signaling cascade. CXCL1-CXCR2 engagement enhances inflammatory responses, increases neutrophil recruitment, and prolongs neutrophil survival by reducing apoptosis[27, 70]. This chemokine signaling promotes the transition of LFA-1 into its high-affinity state[18, 32, 40, 73]. High-affinity LFA-1 is necessary and sufficient to mediate neutrophil arrest through strong binding to endothelial ICAM-1 [32, 33, 45]. Arrested neutrophils subsequently crawl along the endothelial surface in response to ICAM-1 distribution and chemokine gradients before transmigrating into tissues through either paracellular or transcellular route[37, 40, 73]. Efficient clearance of migrated neutrophils is required to restore tissue homeostasis, whereas dysregulated removal or persistent trafficking contributes to chronic inflammation and disease progression[15, 45].

These insights have led to increasing interest in therapeutic approaches that modulate inappropriate or prolonged inflammation responses. Several pharmacological strategies have been proposed, including limiting the production of inflammatory mediators and further neutrophil influx, accelerating the apoptosis of inflammatory cells, enhancing macrophage efferocytosis, and controlling cytotoxic neutrophil effector function[15, 49, 59]. Based on these therapeutic concepts, attention has focused on compounds targeting the adhesion pathway, as these early molecular events largely determine the efficiency and magnitude of neutrophil recruitment. Fuzapladib sodium hydrate (FZP), an LFA-1 inhibitor currently used in veterinary medicine[21], and pentosan polysulfate sodium (PPS), a heparin-like compound known to interfere with selectin-mediated adhesion[11, 22], have been proposed as potential modulators of neutrophil recruitment. Despite their clinical relevance, broader effects of these agents on adhesion molecules and chemokine receptor pathways in canine endothelial cells and neutrophils remain insufficiently defined.

Therefore, this thesis is structured to clarify the dose-dependent modulatory actions of FZP and PPS on the multi-step neutrophil recruitment cascade in a cytokine-stimulated canine cell-based *in vitro* model. To achieve this, a mechanistic approach will be employed: quantitative real-time polymerase chain reaction (qPCR) will be used to determine transcriptional changes in key

selectins (E-selectin, P-selectin), integrin ligands (ICAM-1) on endothelial cells, and neutrophil adhesion molecules (CXCR2, PSGL-1, and L-selectin). Furthermore, the endothelial chemokine, CXCL1 protein, will be evaluated using Enzyme-Linked Immunosorbent Assay (ELISA), and surface integrin LFA-1 (CD11a) expression will be quantified by flow cytometry. These molecular findings will be functionally validated using a static adhesion assay to assess the final outcome on neutrophil adherence to activated endothelial cells. The research is organized into two dedicated chapters: Chapter I is focused on elucidating the effects of FZP, while Chapter II addresses the actions of PPS.

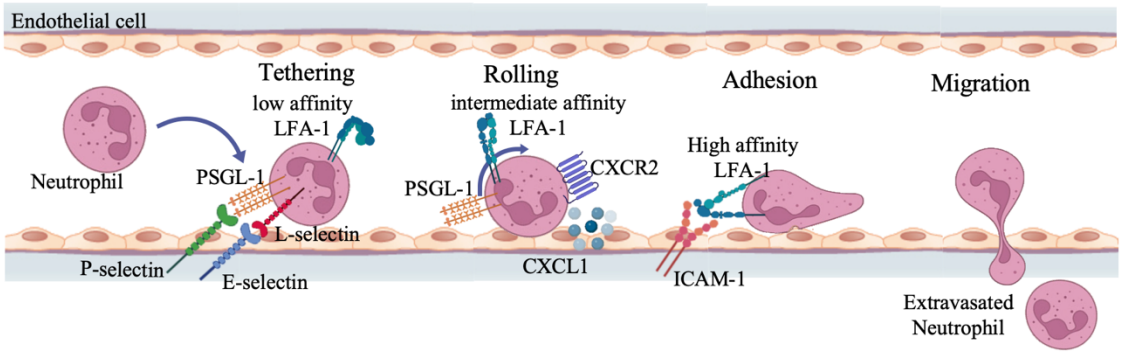

Figure 1. Neutrophil recruitment cascade

The diagram illustrates the sequential steps by which neutrophils exit the bloodstream during inflammation. Initial tethering and rolling are mediated by interactions between endothelial P-/E-selectin, and neutrophil PSGL-1, with L-selectin supporting secondary capture. Exposure to endothelial CXCL1 activates CXCR2 and induces LFA-1 affinity shifts, enabling firm adhesion through binding to ICAM-1. After the arrest, neutrophils transmigrate across the endothelium into the inflamed tissue.

Chapter I

Effects of fuzapladib sodium hydrate on adhesion molecule expression in canine endothelial cells and neutrophils

Summary

Neutrophil recruitment to inflamed tissue is mediated by adhesion molecules and chemokine signaling. FZP has been reported to reduce neutrophil infiltration, but its broader effect on adhesion molecules in dogs remains unclear. This study investigated the influence of FZP on canine aortic endothelial cells (CnAOEC) and primary neutrophils under cytokine stimulation. CnAOEC were treated with recombinant canine tumor necrosis factor alpha (rh-cTNFα) or lipopolysaccharide (LPS) together with FZP, while neutrophils isolated from healthy dogs were preincubated with FZP before rh-cTNFα stimulation. Expression of endothelial E-selectin, P-selectin, and ICAM-1 was quantified by quantitative real-time polymerase chain reaction (qPCR)**.** Neutrophil CXCR2, PSGL-1, and L-selectin were also analyzed using qPCR. In addition, CXCL1 and LFA-1 were determined using Enzyme-linked immunosorbent assay (ELISA) and flow cytometry, respectively. Neutrophil adhesion to endothelial monolayers was evaluated under static conditions. The FZP significantly downregulated endothelial P-selectin at the highest dose, whereas ICAM-1 was upregulated. In neutrophils, FZP decreased L-selectin and PSGL-1 expression at the highest concentration. These findings suggest that FZP selectively modulates early adhesion through suppression of P-selectin, L-selectin, and PSGL-1, which would weaken rolling and tethering interactions between cells. Although functional adhesion effects were limited, these molecular responses provide further insight into the anti-inflammatory effect of FZP.

Introduction

Building on the broader overview of inflammation and neutrophil recruitment presented in the preface, this chapter focuses on the therapeutic modulation of adhesion molecules that coordinate neutrophil-endothelial cell interactions. Neutrophil extravasation is directed by a series of adhesive events occurring where circulating leukocytes and the vascular endothelium intersect. When these adhesive pathways become dysregulated, neutrophils may accumulate excessively in tissues or contribute to systemic inflammatory injury[19]. These observations highlight the importance of regulating the adhesion processes that direct neutrophil recruitment, particularly in conditions where excessive recruitment aggravates inflammation.

Among therapeutic approaches, inhibition of adhesion molecules has been recognized as a promising strategy for limiting neutrophil-mediated inflammation[42, 47]. FZP, an LFA-1 inhibitor, represents one such agent. By interfering with LFA-1-mediated firm adhesion, FZP suppresses neutrophil accumulation and is currently approved for the treatment of canine pancreatitis in the United States of America and Japan[21, 35]. Preclinical studies in rat and canine models have shown that FZP reduces the severity of pancreatic injury by decreasing neutrophil infiltration[23, 24]. Additionally, an *in vitro* study using human umbilical vein endothelial cells (HUVEC) and HL-60 cells showed reduced neutrophil-endothelial adhesion after LPS stimulation and FZP treatment. This effect is commonly associated with a downregulation of LFA-1 expression[57]. Combined, these findings indicate that FZP has the potential to modulate early inflammatory responses through its effects on adhesion pathways.

Despite this evidence, major gaps remain in understanding the broader effects of FZP on the adhesion molecules that mediate neutrophil recruitment. Existing studies have focused primarily on LFA-1, even though neutrophil extravasation depends on multiple adhesion molecules such as E-selectin, P-selectin, ICAM-1, CXCR2, PSGL-1, and L-selectin. These molecules act sequentially during tethering, rolling, firm adhesion, and transmigration[45]. Presently, no study has examined the effects of FZP on these pathways in both endothelial cells and neutrophils within canines. Most available information is derived from rodent models, alongside studies performed in porcine and human cell systems[23, 25, 57, 62, 72]. Given that FZP is clinically used in dogs, clarification of its molecular effects, specifically in canine endothelial cells and primary canine neutrophils, is essential. Furthermore, the dose-dependent characteristics of these responses have not been fully examined.

In this chapter, effects of FZP on the expression of key adhesion molecules involved in neutrophil-endothelial interactions were examined using a canine cell-based *in vitro* model. The analyses include endothelial E-selectin, ICAM-1, and P-selectin; neutrophil CXCR2, PSGL-1, and L-selectin; and the surface expression of LFA-1 across multiple concentrations of FZP. By addressing the lack of data on the broader and dose-dependent effects of FZP in canine inflammatory cells, this chapter provided important insights into its potential mechanism of action and would support more informed clinical use of FZP in conditions characterized by excessive neutrophil recruitment.

Materials and Methods

Cell culture

For this study, CnAOEC (Cat. No Cn304-05; Cell Applications, Inc., San Diego, CA, USA) were obtained as a cryopreserved vial at passage 2. In this study, CnAOEC from Passage 4 to Passage 9 were used. The cells were cultured in the Canine EC Growth Medium (Cat. No. Cn211K-500; Cell Applications) according to the manufacturer's instructions with a minor modification. Briefly, CnAOEC were seeded at a density of 1.0×10^4 cells/cm² onto 100 mm culture dishes (Corning, Lowell, MA, USA) coated with Attachment Factor Solution (AFS; Cat. No. 123; Cell Applications). Fresh medium, pre-warmed to 37 °C, was replaced every other day. When cultures reached 80–90% confluence, cells were washed three times with Hanks' Balanced Salt Solution (HBSS; Cat. No. 14025092; Thermo Fisher Scientific, Waltham, MA, USA) and detached using Acutase solution (Sigma-Aldrich, St. Louis, MO, USA). The cell suspension was then centrifuged at 400 × g for 5 minutes at 4°C without braking, and the cell pellet was resuspended in Canine EC growth medium. Cell number and viability were assessed using the trypan blue exclusion method (Wako Pure Chemical Industries, Osaka, Japan). Following this assessment, cells were expanded and sub-cultured under identical conditions for subsequent experiments.

Canine neutrophil isolation

Ten milliliters of fresh whole blood were obtained from the cephalic vein of three healthy female beagles, with the approval of the Ethics Committee of Hokkaido University (Approval No. 230104). This followed the institutional guidelines for the care and use of experimental animals. Neutrophils were isolated from 10 ml of fresh, anti-coagulated K_2EDTA (Dojindo Laboratories, Kumamoto, Japan) using Polymorphprep (Serumwerk Bernburg, Bernburg, Germany) through density gradient centrifugation, following the manufacturer's protocol for human neutrophil isolation (500 × g for 30 minutes at 20 °C). Residual erythrocytes were eliminated by the addition of 1 ml of 1X RBC lysis buffer (Thermo Fisher Scientific) for 8 minutes at room temperature. This was immediately followed by an addition of HBSS and centrifugation at 400 x g for 10 minutes. The residual neutrophils were resuspended and calibrated to $4x10^6$ cells/ml in the Roswell Park Memorial Institute 1640 medium (RPMI-1640; Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin-streptomycin solution (Wako

Pure Chemical Industries). The isolated neutrophils were immediately used for the subsequent experiment.

RNA extraction, reverse transcription, and quantitative polymerase chain reaction (qPCR)

The CnAOEC were cultured at a density of 2×10^5 cells per well in 6-well plates (Corning), and the Canine EC growth medium was replaced every other day until the cells reached confluence. The cells were subsequently treated in Canine EC Basal Medium (Cat. No. Cn210-500; Cell Applications, Inc.) with either rh-cTNFα (30 ng/ml; Kingfisher Biotech, Saint Paul, MN, USA) or LPS (100 ng/ml; Cat. No. 127-05141; Wako Pure Chemical Industries), each administered alone or in combination with FZP (1, 10, or 100 μM; BRENDA, Ishihara Sangyo Kaisha, Ltd., Osaka, Japan). Treatments were applied for 6 hours in a humidified incubator at 37°C with 5% CO_2. After incubation, the cells were washed three times with HBSS to remove residual medium, and total RNA was then isolated.

Freshly isolated neutrophils, at a concentration of 1×10^6 cells/ml, were pre-incubated with or without FZP at concentrations of 1, 10, or 100 μM in a humidified incubator with 5% CO_2 at 37°C for 24 hours. The cells were then stimulated with rh-cTNFα at 20 ng/ml for 1 hour. RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin served as the negative control, while rh-cTNFα treatment alone was used as the positive control. Following treatment, neutrophils were washed once with phosphate-buffered saline (PBS; Cat. No. 10010023; Thermo Fisher Scientific). Subsequently, the cells were lysed and stored at −70°C until all samples were collected, after which RNA was extracted.

Total RNA was isolated utilizing the NucleoSpin RNA Purification Kit (Macherey-Nagel, Dürren, Germany). The concentration of RNA was determined using a NanoEX Lite spectrophotometer (Optima Inc., Tokyo, Japan) at a wavelength of 260 nm. Subsequently, 500 ng of total RNA from CnAOEC and 50 ng from canine neutrophils were reverse-transcribed into cDNA using the M-MLV RT Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's guidelines. A two-step qPCR was conducted with the KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Woburn, MA, USA). The relative expression levels of target genes were determined using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as the housekeeping gene for normalization. The expression levels of adhesion-related genes, which include E-selectin, ICAM-, P-selectin, CXCR2, PSGL-1, and L-selectin, were evaluated to determine the impact of FZP on endothelial and neutrophil adhesion molecules. Primer sequences

are listed in Table 1[34, 44]. The qPCR assays consisted of three biological replicates for each endothelial cell sample. Moreover, each sample comprised two technical replicates. For neutrophils, the process was as follows. Blood was drawn from a dog on three separate occasions. Each sample was used for two technical replicates. This process was repeated for a total of three dogs.

Enzyme-linked immunosorbent assay (ELISA)

In an independent experiment, CnAOEC were inoculated onto 96-well plates (Corning) coated with AFS at a density of 1×10^5 cells per well in 200 µl of the Canine EC Growth Medium and incubated for 24 hours to allow cell attachment. Following incubation, the culture medium was replaced with one of the following treatments: rh-cTNFα at 30 ng/ml, either alone or combined with FZP at concentrations of 1, 10, or 100 µM, or with LPS at 100 ng/ml, either alone or in combination with FZP (1, 10, or 100 µM). The Canine EC Basal Medium without stimulants or FZP served as the negative control. All procedures were conducted in a humidified incubator at 37°C with 5% CO_2. After 24 hours of stimulation, the concentration of secreted CXCL1 protein in the culture medium was quantified using the Canine CXCL1 ELISA kit (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's instructions.

Flow cytometry

In a separate experiment, neutrophils used in this experiment were isolated from three dogs. For each dog, the experiment was conducted in three separate runs. Fresh isolated neutrophils were subjected to the same pre-incubation and stimulation protocol as described above for the qPCR assay. Following this protocol, the cells were washed once with PBS. Subsequently, 3×10^5 cells were resuspended in PBS containing 10% goat serum (Thermo Fisher Scientific) and incubated at room temperature for 20 minutes to reduce nonspecific antibody binding. The cells were then stained with PE-conjugated mouse anti-human CD11a monoclonal antibody (clone HI111, mouse IgG1; BD Biosciences, San Diego, CA, USA) or PE-conjugated mouse IgG1, κ isotype control (clone MOPC-21; BD Biosciences) for 10 minutes under light-protected conditions. After staining, the cells were washed twice with PBS containing 10% goat serum. Two microliters of 7-Aminoactinomycin D (7-AAD; BD Biosciences) were then added to the stained cell suspension in 200 µl of PBS containing 10% goat serum and subsequently incubated for 10 minutes. Flow cytometric analysis was performed using a BD FACSLyric™ Flow Cytometer (BD Biosciences).

Static adhesion assay

A static adhesion assay, modified from a prior human investigation[69], was conducted to assess the impact of FZP on neutrophil adherence to endothelial cells. Within this experiment, CnAOEC were seeded at a density of 1×10^4 cells per well in 96-well plates. The Canine EC Growth Medium was replaced every other day until the cells reached 100% confluence. Once confluent, the cells were pre-incubated for 3 hours with FZP at doses of 1, 10, or 100 μM, or the Canine EC Basal Medium as a control. Following pretreatment, the cells were exposed to rh-cTNFα at a final concentration of 30 ng/ml and incubated for 4 hours. Freshly isolated neutrophils were labeled with Calcein-AM (Dojindo Laboratories). The labeled neutrophils, at a concentration of 1×10^5 cells per well, were then coincubated with treated CnAOEC for 20 minutes. All incubations were performed at 37 °C in a humidified incubator with 5% CO_2. Fluorescence intensity (FI) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm was measured using a GloMax Discover microplate reader (Promega, Madison, WI, USA). This measurement was performed before the washing step to determine the total cell count. The cells were then gently washed twice with HBSS to remove non-adherent neutrophils. Subsequently, 100 μl of RPMI-1640 solution was added to each well, and fluorescence was re-evaluated. Neutrophil adhesion was calculated as the relative adherence by examining the FI before and after washing using the following equation:

$$\frac{(Prewash\ FI\ of\ sample\ -\ Prewash\ FI\ of\ Background)}{(Postwash\ FI\ of\ sample\ -\ Postwash\ FI\ of\ Background)}\ x\ 100\ =\ \%\ Adherence$$

$$\frac{\%\ Adherence\ of\ treatment}{Average\ \%\ Adherence\ of\ positive\ control}\ =\ Relative\ adherence$$

Similar to the previous experiment, neutrophils were isolated from three healthy dogs, and the experiment was independently repeated three times for each dog. Each repeat included five treatment conditions, with six technical replicates per condition.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 10.0 software (GraphPad Software, San Diego, CA), and a *p*-value < 0.05 was considered statistically significant for all tests. The assumption of a normal distribution was assessed using the Shapiro-Wilk test. A repeated-measures (RM) one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test was used to analyze

normally distributed data. In case the assumption of normality was violated, the Friedman test in combination with Dunn's test was employed instead.

Results

Effect of FZP on adhesion molecule gene expression in cytokine-induced CnAOEC

The qPCR was conducted to assess the effect of FZP on the expression of endothelial adhesion molecules, specifically: E-selectin, ICAM-1, and P-selectin in CnAOEC stimulated with rh-cTNFα or LPS. All expression levels were compared to those of the corresponding positive control. The results indicated that stimulation with both LPS and rh-cTNFα markedly increased the messenger ribonucleic acid (mRNA) levels of all target genes compared to the negative control (data not shown).

Across both stimuli on the CnAOEC for 6 hours, the result illustrated that FZP did not reduce E-selectin transcription level relative to the stimulus control. Under LPS stimuli, the mean fold was set at 1.00, while the treatment groups showed mean values of 0.79, 0.85, and 1.40 for FZP at 1, 10, and 100 μM, respectively, as shown in Figure 2A. None of the doses were statistically significant compared to the LPS control group ($p \geq 0.347$). Among the treatments, the 100 μM group values increased compared to the 1 μM group ($p = 0.027$), yet other pairs were not significant. In the rh-cTNFα-stimulated group (see Figure 3A), the statistics showed no difference between treatment groups versus the positive control ($p \geq 0.395$) and no difference among the treatments ($p \geq 0.534$). The mean folds were 1.00, 0.82, and 0.95 for 1, 10, and 100 μM of FZP, respectively.

ICAM-1 gene expression relative to the positive control exhibits a similar pattern in the LPS-exposed group as E-selectin, illustrated in Figure 2B. The mean fold was 1.00, 0.95, 0.86, and 1.56 for LPS and FZP concentrations of 1, 10, and 100 μM, respectively. The statistical analysis showed no significant effect of the ICAM-1 gene expression after being challenged with all treatments compared to the LPS treatment alone ($p \geq 0.347$). However, ICAM-1 was significantly upregulated in the 100 μM group compared to the 10 μM group ($p = 0.027$). Other pairwise comparisons were not significant. For the rh-cTNFα treatment groups, the mean fold versus rh-cTNFα alone was 0.99, 0.81, and 1.39 at 1, 10, and 100 μM of FZP, respectively, as shown in Figure 3B. These results revealed that FZP at 100 μM concentration significantly increased ICAM-1 gene expression level compared to both the positive control ($p = 0.007$) and the FZP 1 μM treatment group ($p = 0.039$). There were no significant differences between other pairwise comparisons ($p \geq 0.359$).

P-selectin mRNA expression level was highlighted across both stimuli, as it illustrated an inverse-dose response. The results of the LPS-challenged group indicated that the mean fold

against the isolated LPS treatment was 1.09 (1 μM), 1.21 (10 μM), and 0.84 (100 μM), visible in Figure 2C. The statistical calculations showed that 100 μM concentration had a significant effect in reducing P-selectin expression levels compared to the 10 μM group ($p = 0.024$). However, other pairwise comparisons were not statistically significant ($p \geq 0.052$). For the rh-cTNFα treatment groups, the mean fold change compared to the positive control was 1.07, 0.90, and 0.69 for FZP at 1, 10, and 100 μM, respectively, in a dose-dependent pattern. Figure 3C visualizes this pattern. The statistical results displayed that P-selection was downregulated after incubation with FZP 100 μM compared to both incubations with rh-cTNFα alone ($p = 0.009$) and FZP 1μM ($p = 0.046$). However, all remaining pairwise comparisons were not significant ($p \geq 0.157$).

Effect of FZP on CXCL1 protein expression in cytokine-induced CnAOEC

The concentration of CXCL1 protein was quantified using the Canine CXCL1 ELISA kit to determine the effect of FZP on cytokine-induced CnAOEC. The conducted ANOVA test showed no significant effect of treatment on CXCL1 expression in LPS-stimulated samples ($p = 0.286$), illustrated in Figure 4A. On the other hand, there was statistical significance in the overall effect of treatment in the rh-cTNFα-challenged group ($F(1.16, 2.32) = 20.14$, $p = 0.035$), visualized in Figure 4B. However, Tukey's post-hoc comparisons test did not identify a significant comparison between any pair ($p \geq 0.054$).

Effect of FZP on adhesion molecule gene expression in cytokine-induced canine neutrophils

The effect of FZP on neutrophil adhesion molecule gene expression, including CXCR2, PSGL-1, and L-selectin, was evaluated using neutrophils isolated from three healthy dogs. For each dog, experiments were independently repeated three times. Expression values were normalized within the dogs to the rh-cTNFα–stimulated condition. Subsequently, the gene expression data were averaged for each dog before statistical analysis.

The results of this analysis are shown in Figure 5. It highlights that FZP did not significantly affect CXCR2 gene expression across 1, 10, and 100 μM, with relative fold changes of 0.92, 0.80, and 0.88, respectively ($p \geq 0.368$). This is illustrated by Figure 5A. For PSGL-1, expression increased at 1μM (1.40-fold) and declined at 10 and 100 μM (0.94- and 0.53-fold, respectively), visible in Figure 5B. When comparing each result with rh-cTNFα, there was no statistical significance ($p \geq 0.064$). However, 100 μM was significantly lower than 10 μM ($p = 0.037$). L-selectin revealed a similar pattern, showing relative fold changes of 1.81, 0.88, and 0.54 (1, 10,

and 100 μM, respectively). Importantly, expression at 100 μM was significantly lower than that of the rh-cTNFα-stimulated control ($p = 0.005$), as visualized in Figure 5C.

Effect of FZP on LFA-1 on rh-cTNFα-induced canine neutrophils

This study examined the effect of FZP on the surface expression of LFA-1 (CD11a component) in canine neutrophils. Neutrophils were isolated from three dogs, and three independent experiments were performed for each animal. The mean value from each dog was used for statistical analysis. The effect of FZP on the mean fluorescence intensity (MFI) of CD11a is shown in Figure 6A. The mean MFI of the rh-cTNFα control group was 2,818. The FZP-treated groups were 2,892, 2,685, and 2,250 at FZP 1, 10, and 100 μM, respectively. The statistics indicated that there was no significant difference in overall treatment effect ($p = 0.727$). In addition, the flow cytometric analysis also revealed two distinct populations of CD11a-positive cells, characterized by either low or high CD11a expression (Figure 6B). Consequently, the effect of FZP on the CD11a expression population was determined. The mean percentage of live cells with low CD11a expression was 49.59% in the rh-cTNFα-stimulated group and 49.15%, 53.83%, and 66.70% following FZP treatment at 1, 10, and 100 μM, respectively (Figure 6C). Conversely, the proportion of live cells showing high CD11a expression was 37.59%, 47.30%, 42.23%, and 41.41% for the rh-cTNFα control, FZP at 1, 10, and 100 μM groups, respectively, as exhibited in Figure 6D. The results revealed no statistical significance in both the low ($p = 0.253$) and high-expression ($p = 0.608$) populations between any pairs of comparisons.

Effect of FZP on static neutrophil adherence to rh-cTNFα induced CnAOEC

Given the observed downregulation of P-selectin on endothelial cells following treatment with FZP, the potential effect on neutrophil adhesion to rh-cTNFα-stimulated CnAOEC under static adhesion conditions was further investigated. For this experiment, the CnAOEC at passages 4-9 were used. Calcein-AM–labeled neutrophils, isolated from three healthy beagles, were applied to the endothelial cell monolayer following treatment. Three independent experiments were performed for each dog, and the mean per dog was used for statistical analysis.

The results, shown in Figure 7, indicated a marked increase in neutrophil adhesion to CnAOEC in all rh-cTNFα-challenged groups compared to the negative control, the Canine EC Basal Medium (data not shown). Furthermore, the effect of different concentrations of FZP on neutrophil adhesion was evaluated. Overall, there was no statistically significant difference in

adhesion among the treatment groups ($p = 0.446$). A slight numerical reduction in adhesion was observed at 100 μM of FZP compared to the rh-cTNFα positive control. However, this is not statistically significant. Subsequent pairwise comparisons confirmed the lack of significance between any of the groups ($p \geq 0.683$).

Table 1. Primer sequences for qPCR

Primer	Primer sequence	Reference
CXCR2	Forward: 5′ AGCTGCCTTAATCCCCTCAT 3′ Reverse: 5′ CTGAACCTGCTGGGAAACTC 3′	34)
E-selectin	Forward: 5′ GAAATTCTTGGCCCGTCCATC 3′ Reverse: 5′ ATGCAGCCAGCACTTAATCTGAAAC 3′	44)
GAPDH	Forward: 5′ TGTCCCCACCCCCAATGTATC 3′ Reverse: 5′ CTCCGATGCCTGCTTCACTACCTT 3′	44)
ICAM-1	Forward: 5′ CAGGGTTGCCAGGTACAGTT 3′ Reverse: 5′ AGTATGGGCTCAGTGGGTTG 3′	44)
L-selectin	Forward: 5′ GATTTCAACAGGACCAGCAGCA 3′ Reverse: 5′ CCGCAGGGTCCACAAGTAAGA 3′	44)
PSGL-1	Forward: 5′ TTCTGGCTGAGATGGCGATG 3′ Reverse: 5′ AGAGTGGCTGGCTCTGGAGTTC 3′	44)
P-selectin	Forward: 5′ TACAACACCAGCTGTCGCTTCC 3′ Reverse: 5′ GTGCTGTCCACTGTCCCAAGTC 3′	44)

CXCR2: C-X-C motif chemokine receptor 2; GAPDH: glyceraldehyde-3 phosphate dehydrogenase; ICAM-1: intercellular adhesion molecule-1; PSGL-1: P-selectin glycoprotein ligand-1

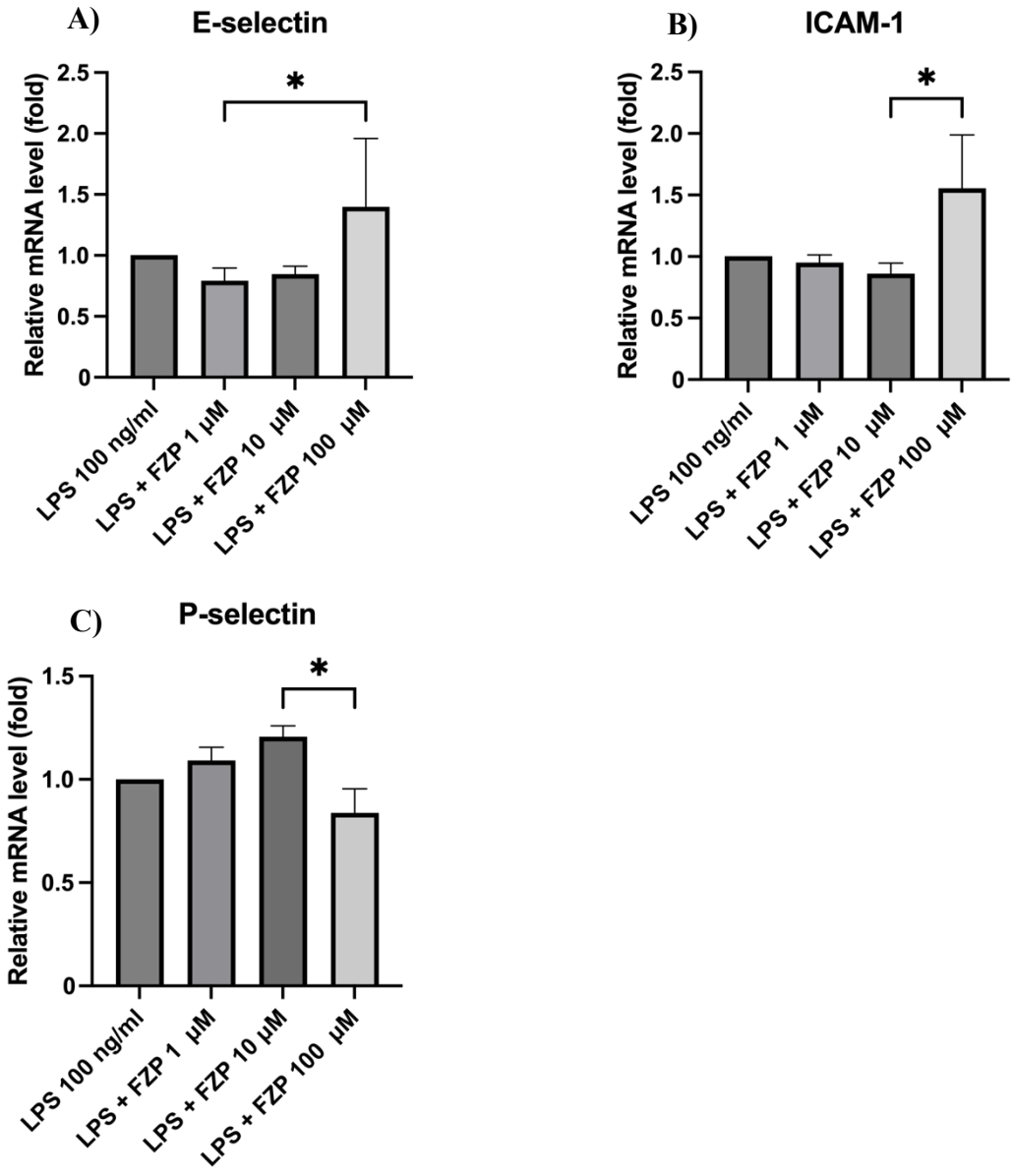

Figure 2. Effects of FZP on adhesion molecule expression in LPS-treated CnAOEC.

CnAOEC were stimulated with LPS (100 ng/ml) alone for 6 hours or in combination with FZP (1, 10, or 100 μM). The mRNA expression of (A) E-selectin, (B) ICAM-1, and (C) P-selectin was analyzed by qPCR and normalized to the LPS control set to 1.0. The data represent the mean ± SD from three independent experiments. Statistical significance was assessed using RM one-way ANOVA with Tukey's post-hoc test, or the Friedman test with Dunn's correction when normality was not met. *$p < 0.05$ versus the indicated groups.

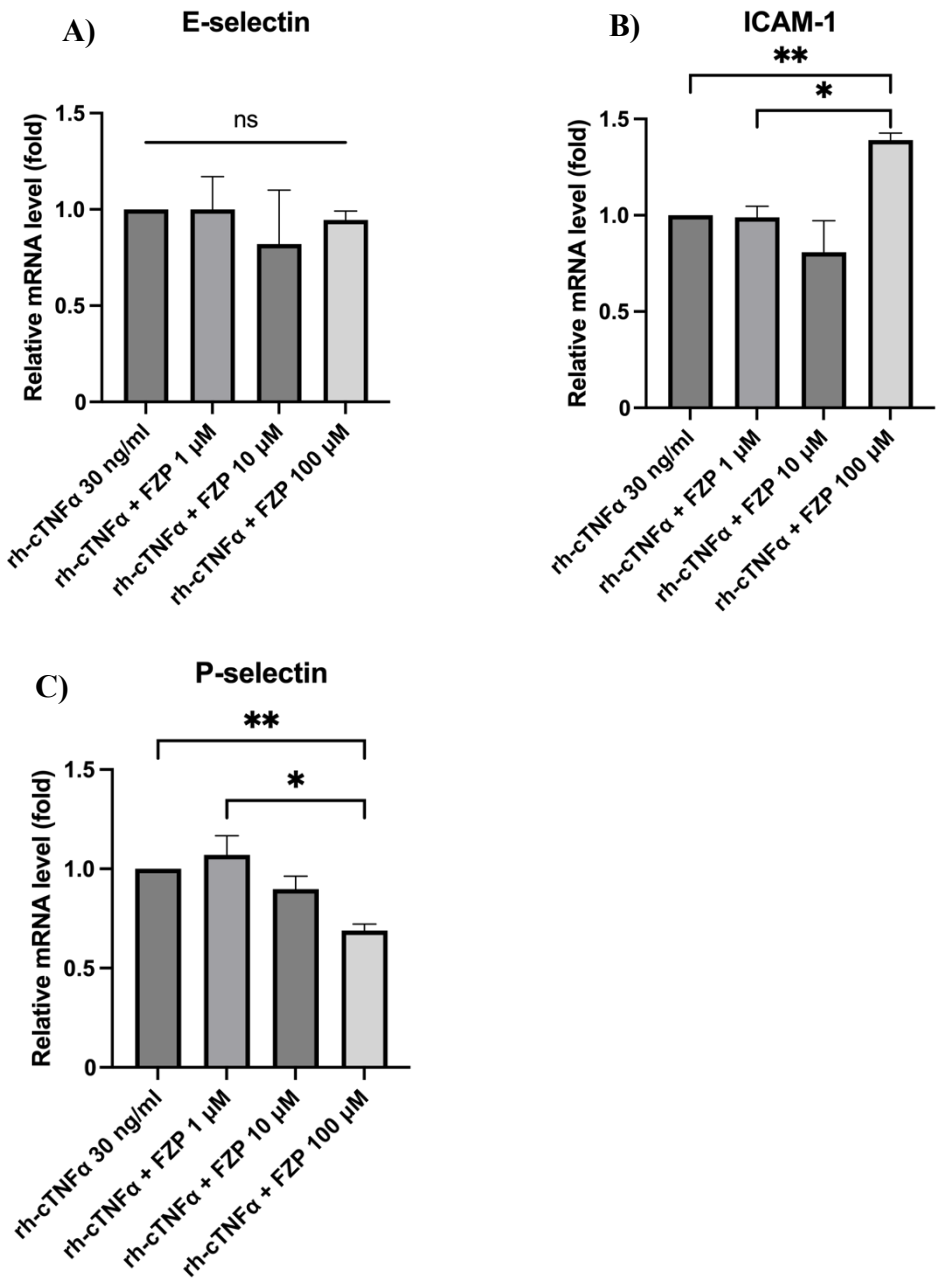

Figure 3. Effects of FZP on adhesion molecule expression in rh-cTNFα-stimulated CnAOEC.

CnAOEC were challenged with rh-cTNFα (30 ng/ml) alone for 6 hours or combined with FZP (1, 10, or 100 μM). The mRNA expression of (A) E-selectin, (B) ICAM-1, and (C) P-selectin was quantified by qPCR and normalized to the rh-cTNFα control (set to 1.0). Data are presented as mean ± SD from three independent experiments. Statistical significance was assessed using RM one-way ANOVA followed by Tukey's multiple comparison test. *$p < 0.05$, **$p < 0.01$ versus the indicated groups.

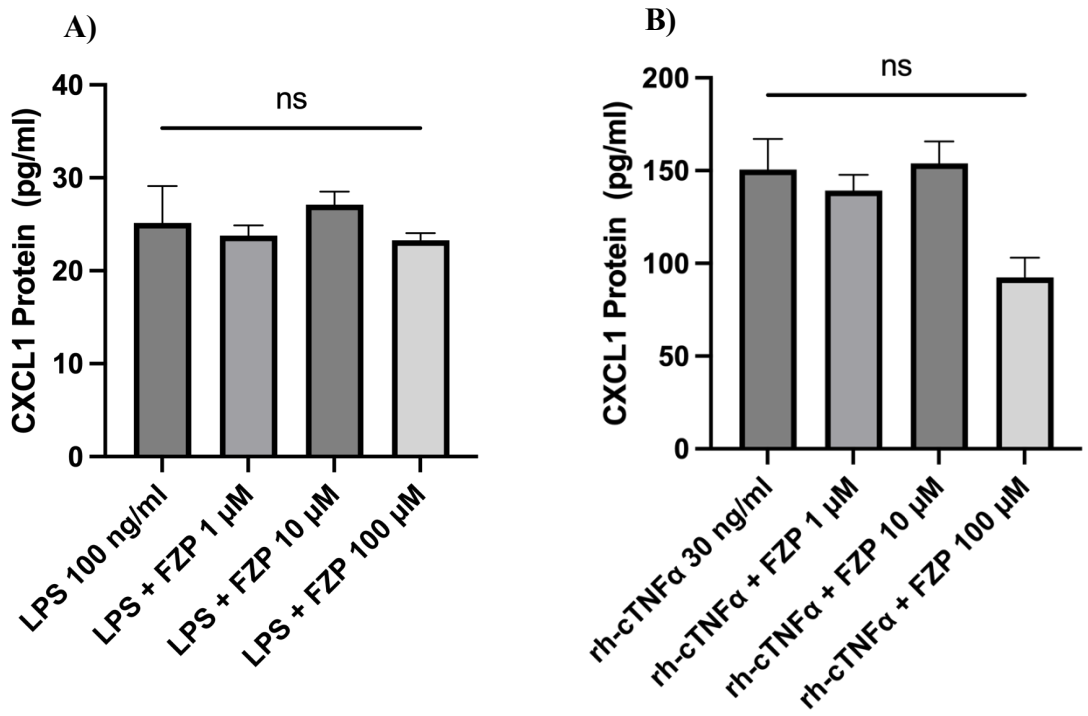

Figure 4. Effects of FZP on CXCL1 protein expression in CnAOEC.

CnAOEC were stimulated with (A) LPS (100 ng/ml) or (B) rh-cTNFα (30 ng/ml), either in isolation or combined with FZP (1, 10, or 100 µM) for 24 hours. CXCL1 levels in culture supernatants were measured using a Canine CXCL1 ELISA kit. Data represent mean ± SD from three independent experiments. Statistical significance was determined by RM one-way ANOVA with Tukey's multiple comparison test ($p > 0.05$).

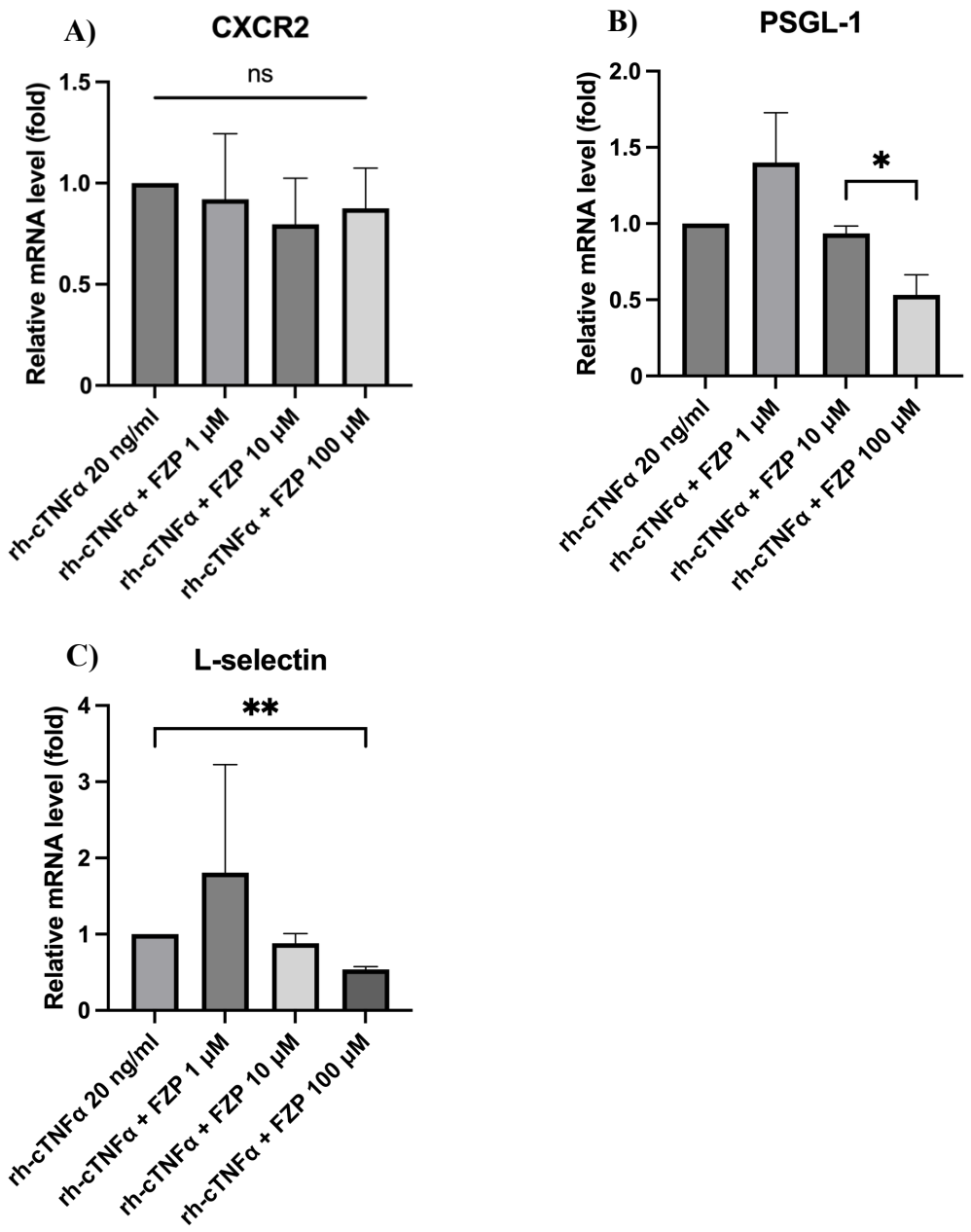

Figure 5. Effects of FZP on adhesion molecule gene expression in cytokine-induced canine neutrophils.

Neutrophils from three dogs were pre-incubated with FZP (1, 10, or 100 μM) for 24 hours and then stimulated with rh-cTNF-α (20 ng/ml) for 1 hour. Gene expression of (A) CXCR2, (B) PSGL-1, and (C) L-selectin was quantified by qPCR and normalized to TNF-α–stimulated controls. Data represent mean ± SD, averaged per dog. Statistical analysis was performed using RM one-way ANOVA with Tukey's test (*$p < 0.05$, **$p < 0.01$).

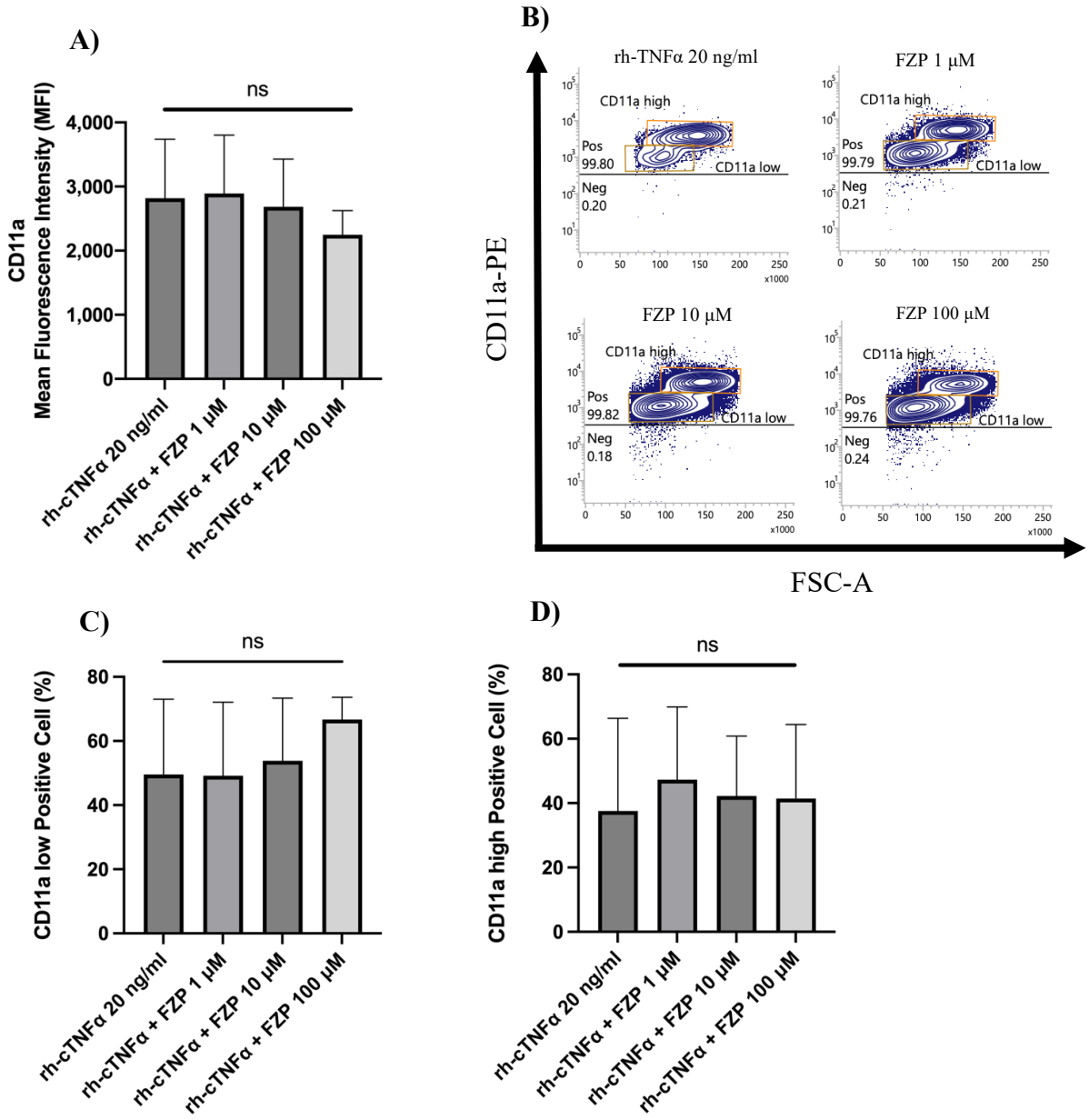

Figure 6. Effects of FZP on LFA-1 (CD11a) expression in rh-cTNFα–stimulated canine neutrophils.

Neutrophils were pre-incubated with FZP (1, 10, or 100 μM) for 24 hours and stimulated with rh-cTNFα (20 ng/ml) for 1 hour. (A) MFI of CD11a expression. (B) Representative flow-cytometric plots showing distinct CD11a low and CD11a high subpopulations. (C, D) Quantitative analysis of low- and high-expression populations, respectively. Each dog was tested in triplicate, using the mean per dog. Data are mean ± SD (n = 3). Statistical significance was assessed using RM one-way ANOVA with Tukey's test, or the Friedman test with Dunn's correction. No significant differences were detected ($p > 0.05$).

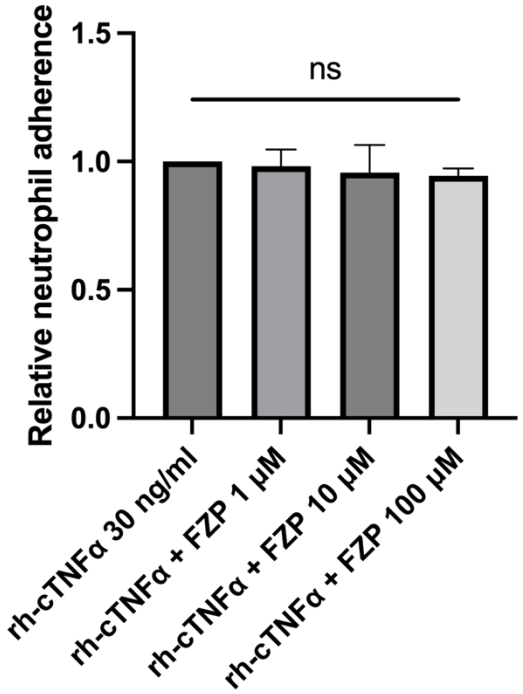

Figure 7. Effect of FZP on static neutrophil adhesion to rh-cTNFα-stimulated CnAOEC.

CnAOEC were pre-incubated with FZP (1, 10, or 100 μM) for 3 hours and subsequently stimulated with rh-cTNFα (30 ng/ml) for 4 hours. Calcein-AM-labeled neutrophils (1×10^5 cells/well) were added and allowed to adhere for 20 minutes. Neutrophil adhesion was quantified as relative fluorescence intensity before and after washing. Data represent mean ± SD from three dogs, each tested in three independent experiments. The mean per dog was used for statistical analysis using the Friedman test with Dunn's post hoc correction, observing no significant effect ($p > 0.05$).

Discussion

This chapter examined the effects of FZP on canine endothelial and neutrophil adhesion molecules relevant to neutrophil recruitment, with a focus on E-selectin, ICAM-1, P-selectin, CXCL1, CXCR2, PSGL-1, L-selectin, and LFA-1. The results indicate that low concentrations of FZP produced non-significant reductions in E-selectin and ICAM-1 gene expression, yet the highest concentration produced an upward shift. These results are partially consistent with a previous study in HUVEC, showing pretreatment with 1 μM FZP did not reduce ICAM-1 protein expression following LPS stimulation[57]. Among endothelial adhesion molecules, P-selectin expression showed a clearer response. In rh-cTNFα-stimulated CnAOEC, FZP caused a dose-dependent reduction, with significant suppression of P-selectin at the highest dose. On the other hand, LPS-stimulated cells displayed mild increases at low concentrations followed by a non-significant decrease. These findings suggest that the influence of FZP on endothelial adhesion molecules may depend on both dose and cytokine context.

The CXCL1-CXCR2 axis, a crucial pathway in neutrophil biology, was also influenced by FZP. CXCL1 is a chemoattractant cytokine that recruits neutrophils through CXCR2, amplifies inflammatory responses, and prolongs neutrophil survival by reducing apoptosis[27], and its dysregulation contributes to the worsening of inflammation, fibrosis, and chronic injury in multiple organs[70]. In this study, both cytokines significantly increased CXCL1 protein levels in CnAOEC. The FZP did not affect CXCL1 secretion under LPS stimulation, but the highest concentration of FZP produced a downward trend under rh-cTNFα, suggesting a possible suppression. Similar effects have been reported previously, in which FZP reduced cytokine-induced neutrophil chemoattractant (CINC, the rat equivalent of CXCL1). These effect were revealed in both *in vitro* (LPS-stimulated bronchoalveolar macrophages) and *in vivo* (cerulein-induced pancreatitis in rats)[72]. Given the critical role of the CXCL1-CXCR2 axis in neutrophil recruitment and tissue injury, particularly in severe pulmonary conditions[54] and in acute pancreatitis where fibroblast signalling further enhances CXCL1 expression[67], even a partial reduction of CXCL1 would have biological relevance by limiting neutrophil infiltration and improving pathology *in vivo* and *in vitro*. The observed reduction in CXCL1 secretion suggests that FZP partly has anti-inflammatory effects in modulating this chemokine axis. This interpretation is further supported by preclinical studies showing reduced macrophage infiltration in the ileal muscularis externa and decreased postsurgical inflammation following FZP treatment[25].

Additionally, clinical data have reported improved outcomes in canine acute pancreatitis, associated with reduced systemic neutrophil extravasation[10].

The functional relevance of these molecular changes was investigated in neutrophil adhesion assays. As expected, rh-cTNFα increased neutrophil adhesion to endothelial cells. Pretreatment of FZP caused a mild, dose-dependent reduction in adhesion that did not reach statistical significance. This suggests that suppression of P-selectin transcription alone is insufficient to inhibit adhesion under static conditions. Previous work has shown that although P-selectin is essential for the initial tethering of neutrophils to endothelial cells, it is inadequate to support firm adhesion without additional signals such as platelet-activating factors[36]. Moreover, P-selectin is implicated in various inflammatory disorders, including dermatitis, lung injury, ischemia, myocardial damage, and arterial thrombosis [13, 39, 64]. Thus, even without a significant change under static conditions, reducing P-selectin would still be relevant *in vivo*, where physiological flow and additional adhesion pathways operate in parallel.

On the neutrophil side, FZP appeared to reduce LFA-1 (CD11a) protein expression in a dose-dependent manner by shifting cells from high to low CD11a expression, leading to an overall decline in MFI. This trend is consistent with earlier suggestions that FZP and related inhibitors can reduce the activation of adhesion molecules such as LFA-1 and MAC-1 in LPS-stimulated HL-60 cells, thus limiting their conformational activation and adhesive function[57]. At high concentrations, FZP also decreased L-selectin and PSGL-1 mRNA expression. L-selectin is essential for leukocyte rolling and secondary tethering between leukocytes[3, 5, 30, 60], while PSGL-1 cooperates with L-selectin to activate LFA-1 and support the transition from rolling to firm adhesion[61]. This results in the reduction of both L-selectin and PSGL-1, suggesting that FZP would affect neutrophil recruitment by disrupting early adhesion. This interpretation is further supported by the established role of L-selectin in directing lymphocyte migration through high endothelial venules[66].

Overall, the results presented in this chapter indicate that FZP modulates several endothelial and neutrophil adhesion molecules in a dose-dependent manner. The most consistent responses were the reduction of P-selectin expression, a downward trend in CXCL1 secretion, and decreased expression of LFA-1, L-selectin, and PSGL-1 at higher concentrations. Although several findings did not reach statistical significance, the overall pattern suggests that FZP influences early neutrophil recruitment through multiple adhesion and chemotactic signals rather than a single dominant pathway. These observations contribute to a clearer understanding of how FZP

modulates neutrophil–endothelial interactions in canine cells and define the scope within which its anti-inflammatory activity could be interpreted in this thesis.

Chapter II

Effect of pentosan polysulfate sodium on adhesion molecule expression in canine endothelial cells and neutrophils

Summary

PPS is a semi-synthetic sulfated polysaccharide with a heparin-like structure, known for its anticoagulant and anti-inflammatory properties. This study investigated the effect of PPS on adhesion molecules involved in neutrophil–endothelial interactions using an *in vitro* canine model. CnAOEC and neutrophils were pretreated with various concentrations of PPS and stimulated with rh-cTNFα. The mRNA expression of E-selectin, ICAM-1, P-selectin, CXCR2, PSGL-1, and L-selectin was measured through qPCR. CXCL1 secreted protein was quantified by ELISA. LFA-1 (CD11a) expression was examined using flow cytometry. A static adhesion assay was used to evaluate the effect of PPS on neutrophil adhesion to endothelial cells. In conclusion, PPS displayed no cytotoxicity toward CnAOEC, and endothelial P-selectin expression was significantly reduced. Other adhesion molecules showed no significant differences compared with controls. Secretion of CXCL1 and neutrophil gene expression (CXCR2, PSGL-1, and L-selectin) varied slightly, although without statistical significance. Yet, PPS did not affect CD11a expression or neutrophil adhesion to endothelial cells. These results indicate that PPS was non-cytotoxic and would weakly modulate selectin-mediated endothelial activation without affecting integrin- or chemokine-dependent adhesion.

Introduction

The previous chapter describes the central processes involved in neutrophil recruitment, providing the foundation for understanding how pharmacological agents may affect adhesive and chemokine-mediated events that initiate neutrophil-endothelial cell interactions. Within that context, this chapter focuses on Pentosan Polysulfate Sodium (PPS), a compound with distinct structural and clinical characteristics that suggest potential relevance for inflammatory modulation.

Pentosan Polysulfate Sodium is a semi-synthetic sulfated polysaccharide derived from beech-wood hemicellulose[20]. It is classified as a low-molecular-weight heparin-like compound with anticoagulant and fibrinolytic activity[58]. In the clinical context, PPS is used across several inflammatory conditions. Furthermore, it is an established treatment for interstitial cystitis[4] and is also used for noninfectious arthritis[17, 29]. In both veterinary and human medicine, PPS is recognized as a disease-modifying osteoarthritis drug (DMOAD) and is used for osteoarthritis and other inflammatory disorders, as well as in the management of thrombotic and atherosclerotic disease[11, 31].

Experimental studies further support anti-inflammatory potential of PPS. In the virus-induced arthritis model, PPS limits cartilage destruction and reduces leukocyte infiltration[20]. Consistent with these findings, PPS has been reported to significantly reduce leukocyte influx[53] and decrease neutrophil accumulation within target tissues[26, 28, 63]. Taken together, these observations indicate a broad potential for PPS to modulate inflammatory cell activity.

At the molecular level, PPS shares structural similarities with endogenous sulfated glycosaminoglycans such as heparin and heparan sulfate[2]. Despite this similarity, PPS shows lower anticoagulant potency than heparin while retaining anti-inflammatory capacity. Importantly, PPS has been reported to interfere with selectin-associated cell adhesion[22], linking its structural characteristics to pathways involved in leukocyte-endothelial interactions. All of these clinical and experimental findings support the possibility that PPS may modulate inflammatory responses by regulating adhesion molecules.

Yet, most existing data on PPS have been conducted in rodents or derived from human clinical research. Extrapolating such findings to dogs is challenging due to inherent species-specific differences in immune responses and endothelial biology[9, 55]. Comparative evidence demonstrates that the regulation of key inflammatory markers, such as P-selectin synthesis and major histocompatibility complex class II expression, differs significantly between murine and

higher mammalian endothelial cells[48]. Consequently, the precise pharmacological impact of PPS on the canine vascular microenvironment remains insufficiently researched. Although the effect of PPS on reducing generalized leukocyte infiltration is well-documented[20, 26, 28, 53, 63], its specific effects on adhesion molecules in canine endothelial cells and neutrophils have not been fully explored. Additionally, dose-dependent responses have not yet been established.

These knowledge gaps serve as the starting point for this study. More specifically, this chapter examines the effects of PPS on the expression of selected adhesion molecules in canine endothelial cells and primary neutrophils across various concentrations. The aim in this chapter is to determine the potential relevance to neutrophil recruitment processes and to investigate the corresponding dose-dependent effects.

Materials and Methods

Canine aortic endothelial Cell culture

Canine aortic endothelial cells (CnAOEC; Cell Applications, Inc.) were obtained as cryopreserved vials at passage 2. For this study, cells from passages 4 to 9 were maintained in the Canine EC Growth Medium (Cell Applications, Inc.) following the manufacturer's protocol with minor adjustments. In short, CnAOEC were seeded at a density of 1.0×10^4 cells/cm^2 in 100 mm culture dishes (Corning) pre-coated with AFS (Cell Applications, Inc.). The 10 ml of fresh prewarmed at 37 °C of the Canine EC Growth Medium was replaced every other day. At 80-90% confluence, CnAOEC were washed with HBSS (Thermo Fisher Scientific) three times, then the cells were detached from the culture plate using Acutase solution (Sigma-Aldrich). The cell suspension was centrifuged at 400 x g for 5 minutes without braking. The cell pellet was resuspended in the Canine EC Growth Medium. Cell count and viability were assessed using the trypan blue exclusion assay (Wako Pure Chemical Industries). Following this assessment, the cells were sub-cultured according to the same established conditions.

Canine neutrophil isolation

Ten milliliters of fresh whole blood were collected from the cephalic vein of three healthy female beagles into a syringe containing K_2EDTA (Dojindo Laboratories). The procedure was approved by the Hokkaido University Ethics Committee (Approval No. 230104) and conducted in accordance with institutional animal care protocols. Neutrophils were isolated from anticoagulated blood by density gradient centrifugation using Polymorphprep (Serumwerk Bernburg), according to the company's instructions for human neutrophil preparation (500 × g, 30 min, 20 °C). Residual erythrocytes were removed by treating the cell pellet with 1 ml of 1× RBC lysis buffer (Thermo Fisher Scientific) for 8 min at room temperature. The cells were then washed with HBSS and centrifuged at 400 × g for 10 min. The neutrophil pellet was resuspended in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin–streptomycin solution (Wako Pure Chemical Industries). Cell count and viability were evaluated using the trypan blue exclusion assay. Freshly isolated neutrophils were immediately subjected to experimental procedures.

Cell viability cell counting kit-8 assay

The CnAOEC were seeded into 96-well plates (Corning) pre-coated with AFS at a density of 5×10^3 cells/100 µl per well in six replicates and incubated in a humidified incubator with 5% CO_2 at 37°C for 24 hours to facilitate cell adhesion. Following incubation, the cells were subjected to treatment with either the Canine EC Basal Medium (Cell Applications, Inc.) as a negative control, pentosan polysulfate sodium (PPS; Oji Pharma Corporation, Ltd., Tokyo, Japan, Lot No: OJI225-01) at concentrations of 1, 10, 50, 80, or 100 µg/ml in the Canine EC Basal Medium, or 10% DMSO (Wako Pure Chemical Industries) as a positive control. Treatments were applied for 24 hours under identical incubation conditions. Cell viability was determined using CCK-8 (Dojindo Laboratories) according to the manufacturer's instructions. Briefly, after the treatment period, 10 µl of CCK-8 reagent was added to each well, and this was followed by a 4-hour incubation period. Cell viability was subsequently evaluated by measuring absorbance at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.). Viability values were normalized to blank controls. All experiments were conducted in three separate trials.

RNA extraction, reverse transcription, and qPCR

The CnAOEC were plated into 6-well plates (Corning) pre-coated with AFS at a density of 2×10^5 cells per well and cultured in Canine Endothelial Growth Medium, which was replaced every other day until the cells reached confluence. Once confluent, the cells were pre-incubated with either the Canine EC Basal Medium alone or supplemented with PPS at concentrations of 1, 10, 50, 80, or 100 µg/ml for 24 hours. After a pre-incubation, the PPS pre-treated groups were exposed to rh-cTNFα (Kingfisher Biotech) at 30 ng/ml. This concentration was selected based on preliminary dose-response experiments on CnAOEC demonstrating maximal endothelial activation while maintaining high cell viability. The positive control group was treated with rh-cTNFα alone, whereas the negative control was maintained in the Canine EC Basal Medium without PPS or in rh-cTNFα. All cultures were incubated for 6 hours at 37 °C in a humidified atmosphere containing 5% CO_2. Following incubation, cells were washed three times with HBSS to remove residual medium, and total RNA was subsequently extracted.

Freshly isolated neutrophils (1×10^6 cells/ml) were pre-incubated with either the RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin solution, either alone or with PPS at concentrations of 1, 10, or 50 µg/ml for 24 hours. After a pre-incubation, the PPS-

treated groups were challenged with rh-cTNFα at 20 ng/ml for 1 hour. The positive control group received only rh-cTNFα, while the negative control was maintained in RPMI-1640 containing 10% FBS and 1% penicillin-streptomycin solution without PPS or rh-cTNFα. Following stimulation, neutrophils were washed once with PBS (Thermo Fisher Scientific). The cells were then lysed and preserved at -70°C until all samples had been collected for subsequent RNA extraction.

Total RNA was extracted using the NucleoSpin RNA Purification Kit (Macherey-Nagel) according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoEX Lite spectrophotometer (Optima Inc.) by measuring absorbance at 260 nm. Subsequently, 500 ng of total RNA from CnAOEC and 50 ng from canine neutrophils were reverse-transcribed into cDNA with the M-MLV Reverse Transcriptase Kit (Invitrogen), following the manufacturer's instructions. The qPCR was performed using the KAPA SYBR FAST qPCR Kit (KAPA Biosystems) in a two-step amplification protocol.

The relative expression levels of target genes were quantified using the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as the internal reference gene for normalization. Adhesion-related genes, including E-selectin, ICAM-1, P-selectin, CXCR2, PSGL-1, and L-selectin, were analyzed to evaluate the effects of PPS on endothelial and neutrophil adhesion molecules. Primer sequences used in this study are listed in Table 1[34, 44]. The qPCR experiments were performed using three independent endothelial cell samples as biological replicates, and each sample was analyzed in duplicate as technical replicates. For neutrophil analysis, blood was collected from each dog on three separate occasions, and each sample was tested twice. This procedure was carried out for three individual dogs.

5. Enzyme-linked immunosorbent assay (ELISA)

In a separate experiment, CnAOEC were plated in 96-well plates at a density of 1×10^4 cells per well in 200 µl of the Canine EC Growth Medium and incubated for 24 hours to allow cell attachment. The cells were then pre-treated with PPS for 24 hours and subsequently stimulated with rh-cTNFα at 30 ng/ml under the same treatment and control conditions as described in the endothelial cell qPCR assay, with a stimulation period of 12 hours. After stimulation, the culture supernatant was collected to quantify the concentration of secreted CXCL1 protein using the Canine CXCL1 ELISA kit (RayBiotech, Inc.) according to the manufacturer's instructions.

Flow cytometry

Freshly isolated neutrophils were subjected to the same pre-incubation and stimulation procedures as those described for the qPCR assay. To evaluate LFA-1 surface expression and verify cell viability under these experimental conditions, cells were gently washed once with PBS and resuspended at 3×10^5 cells in PBS containing 10% goat serum (Thermo Fisher Scientific). The suspension was incubated for 20 minutes at room temperature to minimize nonspecific antibody binding. The cells were then stained with PE-conjugated mouse anti-human CD11a monoclonal antibody (clone HI111, IgG1; BD Biosciences) or with a PE-conjugated mouse IgG1 κ isotype control (clone MOPC-21; BD Biosciences) for 10 minutes in the dark. After staining, the cells were washed twice with PBS containing 10% goat serum. Subsequently, 2 μl of 7-Aminoactinomycin D (7-AAD; BD Biosciences) was added to 200 μl of the stained cell suspension in PBS supplemented with 10% goat serum and incubated for 10 minutes to identify non-viable cells. Flow cytometric analysis was carried out using a BD FACSLyric™ Flow Cytometer (BD Biosciences) to determine the percentage of intact membrane cells (7-AAD negative) within the neutrophil population and the mean fluorescence intensity (MFI). For each dog, the experiment was conducted in three separate runs.

Static adhesion assay

To assess the effect of PPS on neutrophil adhesion to endothelial cells, a static adhesion assay was conducted. This assay is a modification from prior human investigation[69]. Briefly, CnAOEC were seeded in 96-well plates at a density of 1×10^4 cells per well and cultured in the canine EC growth medium. The culture medium was replaced every other day until the cells reached 100% confluence. The confluent monolayers were pre-incubated with PPS at doses of 1, 10, 50, 80, or 100 μg/ml for 3 hours. Next, the cells were challenged with rh-TNF-α at a final concentration of 30 ng/ml for 4 hours. The positive and negative controls were treated under the same established conditions. Freshly isolated neutrophils were labeled with Calcein-AM (Dojindo Laboratories) and added to each well at 1×10^5 cells per well for co-incubation with treated CnAOEC for 20 minutes. All incubation procedures were conducted in a humidified incubator at 37 °C containing 5% CO_2. Fluorescence intensity was measured using a GloMax Discover microplate reader (Promega) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm to quantify the total number of labeled neutrophils before washing. The cells were then

gently washed twice with HBSS to remove non-adherent cells. Subsequently, 100 µl of RPMI-1640 was added to each well for fluorescence re-measurement. Neutrophil adhesion was expressed as the percentage of adherent cells, calculated from the fluorescence intensity values obtained before and after washing, and normalized to the mean adherence observed in the positive control group.

Statistical analysis

All data were expressed as the mean ± SD. Statistical analyses were performed using GraphPad Prism version 10.0 (GraphPad Software), with significance set at $p < 0.05$. Data normality was evaluated using the Shapiro–Wilk test. For normally distributed data, a repeated-measures (RM) one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was applied. When the normality assumption was not met, the Friedman test with Dunn's multiple-comparison test was used.

Result

Effect of PPS on the viability of CnAOEC

To ensure that the observed effect of PPS on the target adhesion molecules was not confounded by cytotoxicity, CnAOEC viability was evaluated using CCK-8 assay after 24 hours of treatment with various PPS concentrations. The negative control group was normalized to 100% cell viability. Following treatment, CnAOEC viability remained high across all tested concentrations, showing 94.8 ± 4.71, 93.4 ± 7.69, 93.5 ± 13.7, 94.6 ± 16.8, and 96.2 ± 11.9 % cell viability for PPS 1, 10, 50, 80, and 100 μg/ml, respectively, as shown in Figure 8. A one-way ANOVA test revealed no statistically significant difference in cell viability among the control and all PPS-treated groups (p = 0.974). These results illustrate that PPS, at the concentrations and exposure duration used in this study, does not compromise the viability of CnAOEC.

Effect of PPS on adhesion molecule gene expression in rh-cTNFα-stimulated CnAOEC

The expression of endothelial adhesion molecules, including E-selectin, ICAM-1, and P-selectin, in CnAOEC stimulated with rh-cTNFα was quantified by qPCR to determine the effect of PPS treatment. All expression levels were normalized to the corresponding positive control. Stimulation with rh-cTNFα markedly increased the mRNA expression of all target genes compared with the negative control. Stimulation with rh-cTNFα induced a marked inflammatory response compared with the negative control, resulting in a 379 ± 95.4-fold increase in E-selectin, a 30.5 ± 4.33-fold increase in ICAM-1, and a 3.30 ± 0.32-fold increase in P-selectin mRNA expression (p < 0.05).

The relative mRNA expression of E-selectin was calculated as a fold change relative to the positive control (set at 1.00). Treatment with PPS at 1, 10, and 80 μg/ml resulted in slightly increased fold changes of 1.25 ± 0.12, 1.02 ± 0.24, and 1.18 ± 0.74, respectively. On the other hand, 50 μg/ml (0.88 ± 0.16) and 100 μg/ml (0.84 ± 0.32) showed mild downregulation. However, these differences were not statistically significant among the groups (p = 0.505) (Figure 9A).

The ICAM-1 mRNA expression was also quantified as a fold change relative to the positive control (set at 1.00). The mean fold changes for PPS at 1, 10, 50, 80, and 100 μg/ml were 1.17 ± 0.13, 1.23 ± 0.17, 1.11 ± 0.08, 1.24 ± 0.38, and 1.06 ± 0.12, respectively (Figure 9B). Despite minor fluctuations across concentrations, no statistically significant differences were observed within the groups (p = 0.482).

In contrast, P-selectin expression showed a general decreasing trend in most PPS-treated groups compared with the positive control (set at 1.00). The 1 μg/ml group demonstrated a visible reduction (0.95 ± 0.01), which was statistically significant ($p = 0.044$). Slight decreases were also observed at 10, 50, and 100 μg/ml, with fold changes of 0.99 ± 0.36, 0.80 ± 0.38, and 0.94 ± 0.27, respectively, although these differences were not significant. In contrast treatment with 80 μg/ml PPS resulted in a mild increase (1.52 ± 1.18), but this change was not statistically significant (Figure 9C).

Effect of PPS on CXCL1 protein expression in rh-cTNFα-induced CnAOEC

Following 24 hours of PPS pretreatment and subsequent rh-cTNFα stimulation for 12 hours, the CXCL1 concentration in the culture supernatant was quantified using a canine CXCL1 ELISA kit to evaluate the effect of PPS on CXCL1 protein expression. The mean CXCL1 concentration showed only slight variation among the PPS-treated groups, with values of 32.23 ± 3.45, 33.40 ± 4.13, 33.70 ± 5.57, 35.86 ± 5.65, and 35.78 ± 6.19 pg/ml for 1, 10, 50, 80, and 100 μg/ml, respectively, compared with 32.90 ± 4.96 pg/ml in the rh-cTNFα control group (Figure 10). Note that one group was not normally distributed. Consequently, data were analyzed using the Friedman test followed by Dunn's multiple comparisons test. The Friedman test indicated a statistically significant overall difference among the treatment conditions ($\chi^2 = 11.19$, $p = 0.018$). However, Dunn's post-hoc analysis revealed no significant pairwise differences in CXCL1 secretion ($p \geq 0.132$).

Effect of PPS on adhesion molecule gene expression in cytokine-induced canine neutrophils

The effect of PPS on the expression of neutrophil adhesion molecule genes, including CXCR2, PSGL-1, and L-selectin, was investigated using neutrophils isolated from three dogs. Each experimental condition was conducted in triplicate for every donor. Neutrophil viability under the incubation conditions used for the gene expression assays was evaluated by 7-AAD staining in parallel flow cytometry experiments performed under identical protocols. High membrane integrity was maintained across all treatments (90.6–91.6%), with no significant differences between groups ($p \geq 0.094$). Gene expression levels were normalized to the corresponding rh-cTNFα-stimulated control condition, which was set as 1.00 within each dog, and the mean values were used for statistical analysis. As shown in Figure 11, PPS treatment produced only minor variations in the expression of the three genes. The CXCR2 (Figure 11A) mRNA

expression slightly increased in fold change at 1 μg/ml (1.19 ± 0.47) and 50 μg/ml (1.14 ± 0.39), whereas a small reduction was observed at 10 μg/ml (0.85 ± 0.13). However, these variations were not statistically significant ($p = 0.427$). For PSGL-1 (Figure 11B), mRNA expression was modestly lower across all PPS-treated groups, with fold-change values of 0.92 ± 0.18, 0.92 ± 0.19, and 0.98 ± 0.18 for 1, 10, and 50 μg/ml, respectively. These differences were also not statistically significant ($p = 0.605$). Meanwhile, L-selectin (Figure 11C) mRNA expression showed a gradual decrease with increasing PPS concentration, yielding fold-change values of 1.01 ± 0.14, 0.88 ± 0.11, and 0.87 ± 0.09 for 1, 10, and 50 μg/ml, respectively. Nevertheless, these variations did not reach statistical significance ($p = 0.154$). Collectively, these results indicate that PPS exposure did not significantly influence the expression of CXCR2, PSGL-1, or L-selectin in rh-cTNFα-stimulated canine neutrophils, although a slight tendency toward downregulation was observed.

Effect of PPS on LFA-1 expression on rh-cTNFα-stimulated canine neutrophils

This study investigated the effect of PPS on the surface expression of CD11a, the α-subunit of LFA-1, in canine neutrophils. Neutrophils were obtained from three dogs, with three independent experiments performed per dog. The mean value for each dog was used for statistical analysis. The MFI of CD11a is presented in Figure 12. The rh-cTNFα control group had an MFI of 2,775 ± 911, while the PPS-treated groups exhibited slightly lower values of 2,547 ± 782 at 1 μg/ml, 2,652 ± 867 at 10 μg/ml, and 2,538 ± 804 at 50 μg/ml. Despite this minor reduction, statistical analysis indicated no significant difference in CD11a surface expression among the experimental conditions ($p = 0.283$).

Effect of PPS on static neutrophil adherence to rh-cTNFα-induced CnAOEC

Given the observed downregulation of P-selectin on endothelial cells following treatment with PPS, the potential effect on neutrophil adhesion to rh-cTNFα-stimulated CnAOEC under static adhesion conditions was further investigated. CnAOEC at passages 4-9 were used for this experiment. Calcein-AM–labeled neutrophils, isolated from three beagles, were applied to the endothelial cell monolayer following treatment. Three independent experiments were performed for each dog, and the mean per dog was used for statistical analysis.

The results indicated a marked increase in neutrophil adhesion to CnAOEC in the rh-cTNFα-challenged positive control groups compared to the negative control, showing a 5.53 ± 1.38-fold increase in adherence ($p < 0.05$). Furthermore, the effect of different concentrations of

PPS on neutrophil adhesion was evaluated. Overall, there was no statistically significant difference in adhesion among the treatment groups (p = 0.276). The results are visible in Figure 13. The relative neutrophil adhesion mean values were 1.00 ± 0.04 (PPS 1 µg/ml), 0.95 ± 0.01 (PPS 10 µg/ml), 0.97 ± 0.03 (PPS 50 µg/ml), 0.97 ± 0.08 (PPS 80 µg/ml), 1.02 ± 0.06 (PPS 100 µg/ml) relative to the rh-cTNFα group set as 1.00. Data were analyzed using the Friedman test. The statistical analysis revealed no significant difference across all conditions (p = 0.276).

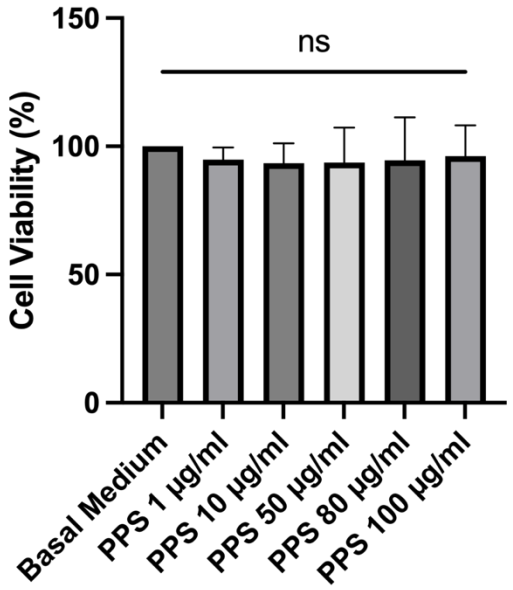

Figure 8. Effect of PPS on the viability of CnAOEC.

CnAOEC were treated with various concentrations of PPS (1, 10, 50, 80, and 100 µg/ml) for 24 hr, and cell viability was assessed using a CCK-8 assay. Data are presented as the mean ± SD from three independent experiments and were analyzed using RM one-way ANOVA. No statistically significant differences were observed among PPS-treated groups and the control ($p > 0.05$).

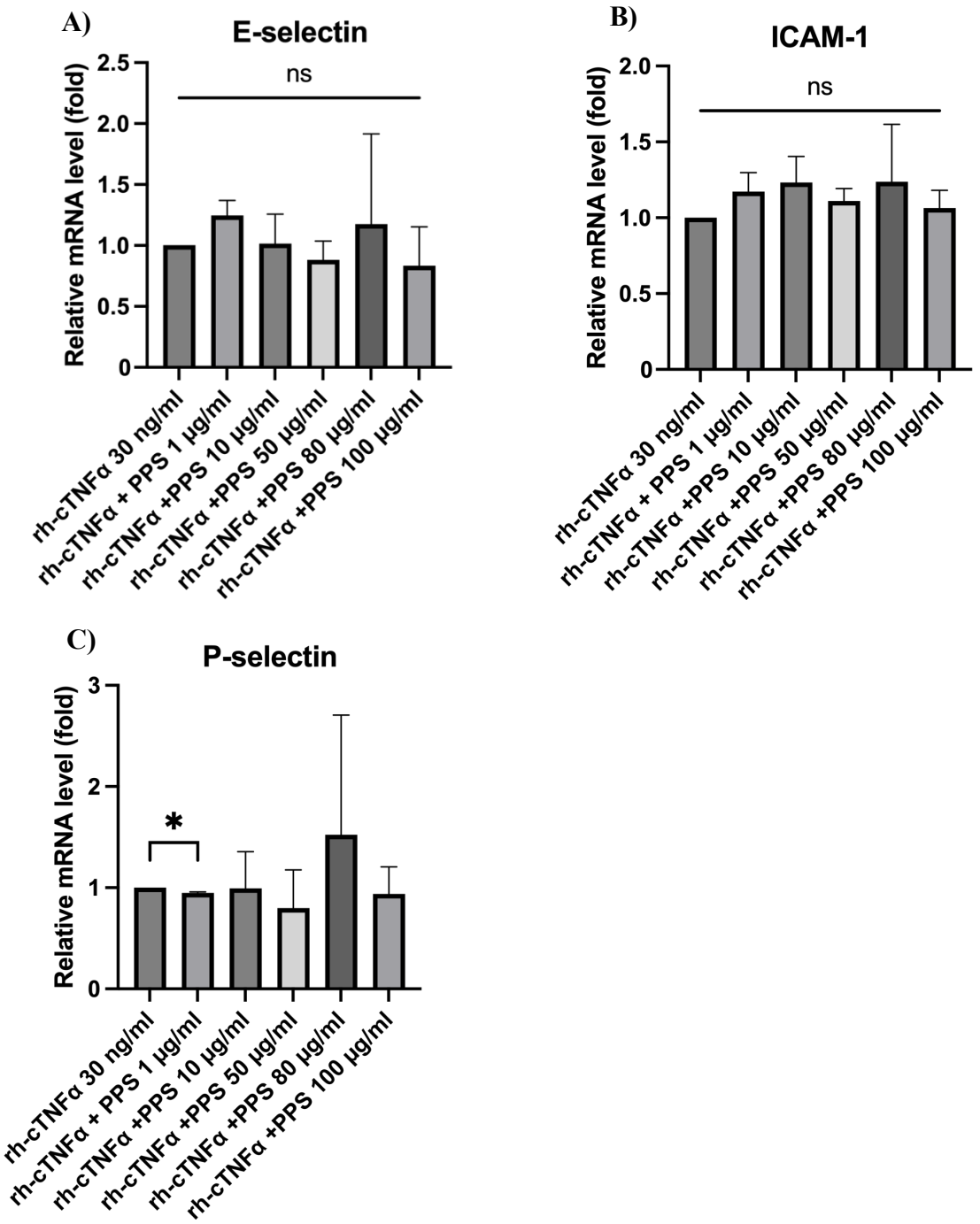

Figure 9. Effects of PPS on adhesion molecule expression in rh-cTNFα-treated CnAOEC.

CnAOEC were subjected to different concentrations of PPS (1, 10, 50, 80, and 100 μg/ml) for 24 hr and subsequently stimulated with rh-cTNFα for 6 hr. The expression levels of (A) E-selectin, (B) ICAM-1, and (C) P-selectin were quantified by qPCR and normalized to the rh-cTNFα-treated positive control (set at 1.00). Bars represent mean ± SD (n = 3). RM one-way ANOVA revealed a significant decrease in P-selectin expression at 1 μg/ml PPS ($p < 0.05$).

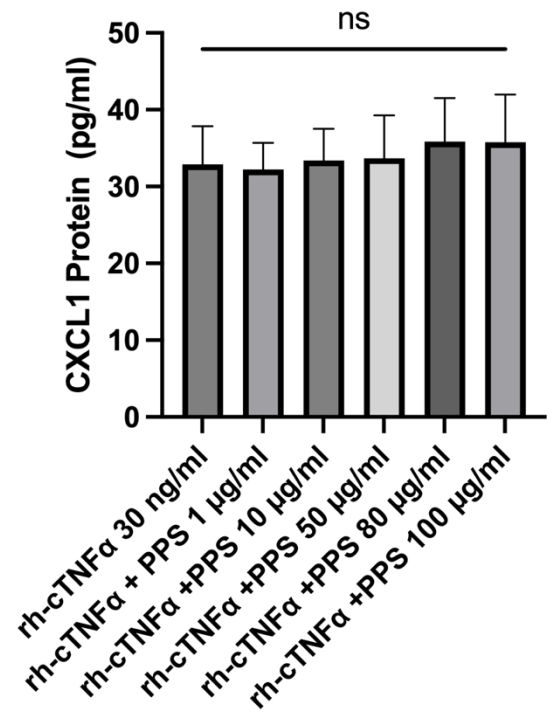

Figure 10. Effect of PPS on CXCL1 protein expression in rh-cTNFα-stimulated CnAOEC.

CnAOEC were exposed to PPS (1, 10, 50, 80, and 100 μg/ml) for 24 hr, followed by stimulation with rh-cTNFα for 12 hr. The CXCL1 concentration in the culture supernatant was measured using a canine CXCL1 ELISA kit. Data were analyzed using the Friedman test with Dunn's post-hoc correction. Values shown are mean ± SD (n = 3).

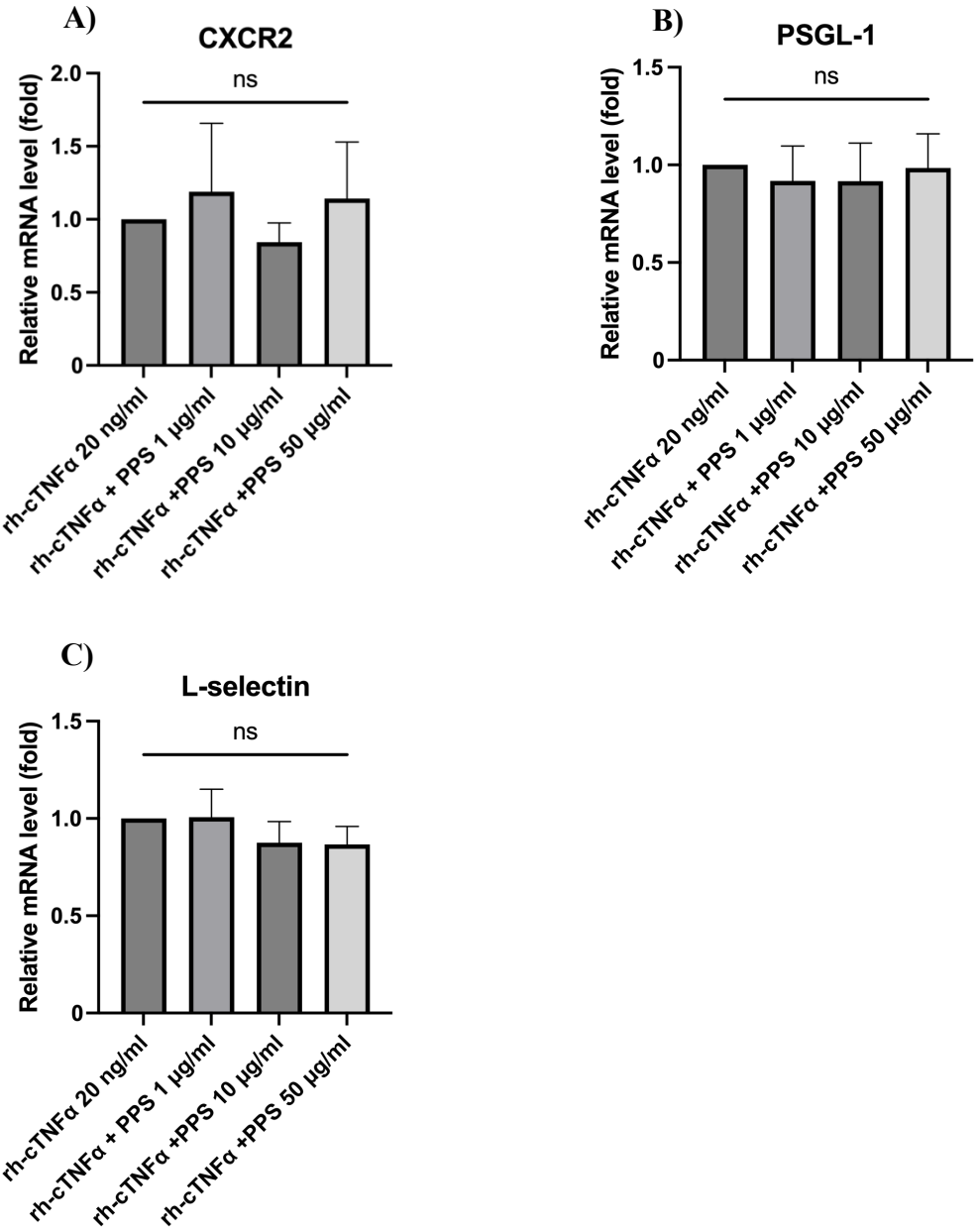

Figure 11. Effect of PPS on neutrophil adhesion molecule gene expression in rh-cTNFα-stimulated canine neutrophils.

Neutrophils isolated from three clinically healthy dogs were treated with PPS at concentrations of 1, 10, and 50 μg/ml for 24 hr, followed by stimulation with rh-cTNFα for 1 hr. The relative mRNA expression levels of (A) CXCR2, (B) PSGL-1, and (C) L-selectin were quantified by qPCR and normalized to the rh-cTNFα-stimulated control (set as 1.00). Data are presented as mean ± SD (n = 3). Statistical analysis using repeated-measures one-way ANOVA showed no significant differences among treatment conditions ($p > 0.05$ for all genes).

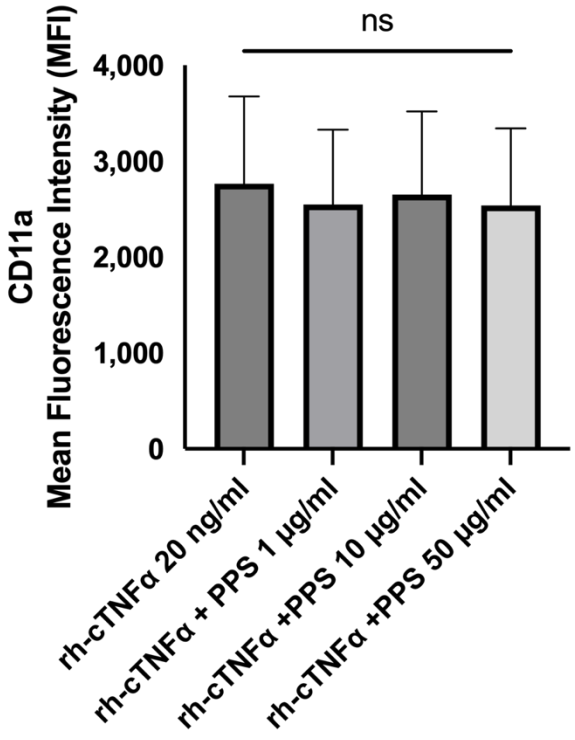

Figure 12. **Effect of PPS on LFA-1 expression on rh-cTNFα-stimulated canine neutrophils.**

Canine neutrophils were pretreated with PPS at concentrations of 1, 10, and 50 µg/ml for 24 hr, subsequently stimulated with rh-cTNFα (20 ng/ml) for 1 hr. The expression of CD11a, the α-subunit of LFA-1, was evaluated by flow cytometry. Data are shown as mean ± SD (n = 3). Statistical analysis by RM one-way ANOVA found no significant difference among treatment groups ($p > 0.05$).

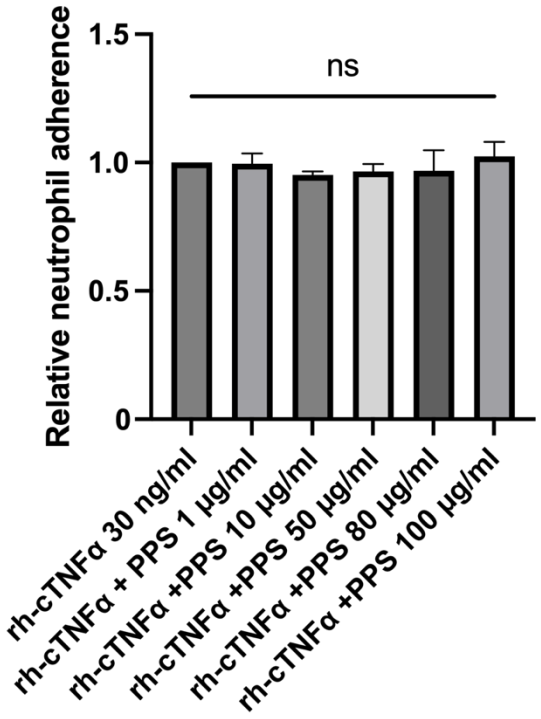

Figure 13. Effect of PPS on static neutrophil adhesion to rh-cTNFα-stimulated CnAOEC.

CnAOEC were pretreated with PPS (1, 10, 50, 80, and 100 μg/ml) for 3 hr, followed by stimulation with rh-cTNFα (30 ng/ml) for 4 hr. Calcein-AM–labeled neutrophils (1×10^5 cells/well) were added and allowed to adhere for 20 min. Neutrophil adhesion was quantified as relative fluorescence intensity before and after washing. Data represent the mean ± SD from three dogs, each tested in three independent experiments, and the mean per dog was used for statistical analysis. Statistical analysis using the Friedman test with Dunn's post hoc correction showed no significant differences among treatment groups ($p > 0.05$).

Discussion

The present study aimed to investigate the effects of PPS on key adhesion molecules, specifically E-selectin, ICAM-1, P-selectin, CXCL1, CXCR2, PSGL-1, L-selectin, and LFA-1, and to examine dose-dependent responses in an *in vitro* canine base model. The results confirmed that PPS at 1-100 µg/ml for 24 hours was non-cytotoxic to CnAOEC, indicating good cellular tolerance under these treatments. This experimental setup aligns with previous in vitro studies, where PPS has been applied at 1-200 µg/ml. Reported exposure durations typically range from 6 to 72 hours across various cell types to evaluate toxicity, permeability, or anti-inflammatory effects[6, 12, 65, 68]. Furthermore, PPS has been reported to modulate gene and protein expression in mesenchymal precursor cells within 24-48 hours of exposure, further supporting the chosen incubation time. Overall, these comparisons support that the conditions used in this study are consistent with established experimental parameters known to balance potential biological activity with cellular safety. This aligns with previous findings that PPS generally could maintain cell viability at therapeutic concentrations, with cytotoxicity usually associated with significantly higher concentrations, prolonged exposure, and elevated sulfate levels[1, 43, 68].

Pentosan Polysulfate Sodium is a low molecular weight heparin-like compound, known for its anticoagulant, anti-adhesive, and anti-inflammatory properties[58]. Due to its structural similarity to heparin, it can competitively interfere with selectin-mediated cell adhesion by inhibiting the Selectin family[58]. Heparin has been reported as an effective P-selectin and L-selectin inhibitor, mediating neutrophil tethering and rolling by PSGL-1. However, it was not effective against E-selectin *in vitro* and inhibited neutrophil accumulation in acute inflammation *in vivo*[41]. In addition, heparan sulfate on endothelial cells helps maintain local chemokine gradients, such as CXCL1, that guide neutrophil migration[46]. Considering these structural and functional similarities, this study supports the potential inhibitory effect of PPS on the targeted adhesion molecules.

In this study, PPS reduced endothelial P-selectin mRNA expression across the tested concentrations, with a significant difference observed at 1 µg/ml. This finding matches previous evidence that PPS is recognized as an oral P-selectin blocking agent used to treat sickle cell disease[31]. The PPS has also been reported to reduce myocardial infarction size by limiting neutrophil accumulation through suppression of terminal complement proteins (C5a and C5b-9)[26, 28, 63]. These are essential for mediating P-selectin expression by promoting rapid P-selectin translocation from the intracellular to the cell surface[50]. Interestingly, the inhibitory effect on P-

selectin mRNA was significant at 1 μg /ml but diminished at higher concentrations. This non-linear response has been observed with other sulfated polysaccharides, in which biological activity often depends on specific charge densities and structural conformations rather than concentration alone[22]. At higher doses, excessive negative charge density may lead to non-specific electrostatic repulsion, interfering with the specific binding interactions required to modulate selectin expression. Thus, 1 μg/ml may represent the optimal therapeutic window for targeting endothelial P-selectin in this model.

E-selectin expression showed a non-significant reduction at higher doses (50 and 100 μg/ml), suggesting a weaker regulatory effect compared with P-selectin. This finding is consistent with an earlier report showing that PPS mainly interferes with P-selectin-mediated cell rolling, while having no effect on E-selectin-dependent rolling[22]. On the other hand, ICAM-1 expression was upregulated across PPS concentrations, indicating that PPS may not suppress the integrin-mediated firm adhesion phase. This observation differs from earlier reports in other cell types, showing that PPS decreased ICAM-1 and CXCL1 mRNA expression in TNFα-induced mice proximal tubular cells[71], and reduced soluble ICAM-1protein level in mice lung[28]. Although CXCL1 protein levels in this study varied not vary statistically, the small decrease observed at 1 μg/ml may indicate a weak inhibitory effect of PPS. This observation is supported by a previous investigation demonstrating that PPS reduced CXCL1 cytokine levels in Chikungunya virus-infected mice, which was associated with reduced neutrophil infiltration[52].

In neutrophils, PPS exposure did not produce statistically significant changes in the expression of adhesion-related molecules. Nevertheless, PSGL-1, L-selectin, and LFA-1 showed a consistent tendency toward lower expression across all PPS-treated groups. These results imply suppression of molecules involved in the neutrophil tethering and rolling step, as the PSGL-1-L-selectin complex provides an essential signal for neutrophil rolling[61], as well as the change from low to intermediate affinity of LFA-1 also regulates this process[18]. In contrast, CXCR2 expression showed only minor variation, indicating that PPS may have less influence on chemokine-receptor–mediated activation.

These findings suggest that PPS has a limited impact on neutrophil activation but potentially weakly modulates the selectin-mediated rolling phase during the early stage of adhesion. This interpretation aligns with previous studies reporting that sulfated polysaccharides, including heparin and PPS, can mimic natural glycosaminoglycans and competitively interfere

with selectin–ligand interactions, reducing neutrophil tethering and rolling[22, 41]. The lack of significant changes in the static adhesion assay supports this interpretation. This result is reasonable, since static conditions mainly represent the firm adhesion stage dominated by the LFA-1/ICAM-1 interaction, while the selectin-mediated rolling phase cannot be clearly reproduced. Therefore, the effect of PPS on selectin-dependent adhesion appears to be specific to the rolling phase, which might be more evident under physiological flow conditions.

However, several limitations of this study should be noted. All experiments were conducted under static *in vitro* conditions, which may not fully reflect the dynamic flow and complex cell interactions that occur *in vivo*. Additionally, while neutrophil recruitment predominantly occurs in post-capillary venules[33], CnAOEC were used in this study as a standardized canine macrovascular model to ensure reproducibility. This approach aligns with previous investigations that employed macrovascular endothelial cells, such as canine jugular vein endothelial cells[13] and HUVEC[57], to define fundamental adhesion mechanisms. Future studies incorporating canine microvascular endothelial cells could further validate these findings regarding tissue-specific responses. The number of donor dogs and the tested PPS concentrations were limited, which may have affected variability and statistical power. Moreover, most analyses focused on gene and surface expression, while post-translational modifications and intracellular signaling were not examined in detail. In the adhesion assay, PPS exposure was mainly tested in endothelial cells, and the direct response of neutrophils was not separately evaluated. Future studies using a flow-based adhesion system and co-culture models under cytokine stimulation, combined with a larger sample size and protein-level analyses, are needed to confirm these findings and clarify how PPS modulates neutrophil-endothelial cell interactions.

In summary, this chapter demonstrates that PPS exerts potential anti-inflammatory effects on canine endothelial cells, primarily by downregulating P-selectin expression. While it showed limited effects on firm adhesion molecules (ICAM-1) or static adhesion, the specific suppression on P-selectin aligns with the mechanism of heparin-like compounds in targeting the early tethering and rolling phase of the leukocyte adhesion cascade. These findings could provide a foundation for understanding the immunodulatory role of PPS in canine *in vitro* model.

Conclusion

This thesis examines the modulatory effect of two clinically relevant anti-inflammatory agents, FZP and PPS, on key adhesion molecules and chemokines that regulate the neutrophil recruitment cascade, particularly in a canine cell-based model. Gene expression, protein levels, and static adhesion function were analyzed in this study. This thesis provides information on how FZP and PPS influence the adhesion molecules that regulate the early step of inflammation.

In Chapter I, FZP demonstrated the ability to downregulate P-selectin at the transcription level in a dose-dependent manner. However, the effect on E-selectin and ICAM-1 was limited. At the protein level, FZP suppressed CXCL1 protein secretion from CnAOEC at a high concentration, though this did not reach statistical significance. On the neutrophil side, FZP reduces L-selectin and PSGL-1 mRNA expression at the highest dose. It also suppresses LFA-1 (CD11a subunit) expression by shifting the high-expression population toward the low-expression population. Collectively, these results suggest that FZP modulates the tethering and rolling steps rather than the firm adhesion process. This explains why FZP did not significantly inhibit the static adhesion assay, which predominantly measures the firm adhesion step. Overall, the findings refine the mechanistic understanding of FZP by showing that its influence extends beyond LFA-1 inhibition and includes selectin-associated pathways.

In Chapter II, PPS shows a more selective pattern of modulation. The PPS consistently decreases P-selectin mRNA expression, whereas E-selectin, ICAM-1 mRNA, and CXCL1 protein levels were minimally affected. In primary canine neutrophils, adhesion molecule mRNA showed only mild fluctuations, and CD11a surface protein remained unchanged. Importantly, this study confirmed that PPS was non-toxic across all treatment concentrations. Similar to FZP, the functional static adhesion assay showed no significant inhibition, confirming that PPS targets the selectin-mediated tethering and rolling phase rather than the integrin-mediated firm adhesion phase.

Although these two chapters were conducted individually, a quantitative comparison reveals underlying differences in their mechanisms. The PPS demonstrated remarkable potency regarding endothelial modulation, achieving significant downregulation of P-selectin at a concentration as low as 1 μg/ml. However, its effect remained highly specific to the endothelium; even at a concentration up to 100 μg/ml, PPS did not significantly alter neutrophil adhesion molecule expression.

In contrast, FZP exhibited a broader range of activity. While it required a higher mass concentration (100 µM, approximately 40 µg/ml) to achieve significance, FZP successfully downregulated adhesive targets on both the endothelium (P-selectin) and the neutrophil (L-selectin, PSGL-1). This suggests that PPS functions as a highly potent, endothelium-specific stabilizer, whereas FZP exerts a comprehensive blockade of the rolling complex on both cell types.

Overall, the thesis contributes conceptual and mechanistic insight relevant to the clinical use of FZP and PPS in dogs. It clarifies the extent of their influence on early neutrophil recruitment and delineates the differences in their anti-inflammatory profiles. Fuzapladib appears to exert broader effects across multiple adhesive pathways, whereas PPS demonstrates mild and selectin-focused modulation. These findings support more informed therapeutic interpretation and provide a scientific foundation for designing future work using flow chambers, co-culture systems, protein-level and post-translational analyses, and ultimately *in vivo* validation.

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Summary in Japanese

好中球の遊走は、セレクチン、インテグリン、およびケモカイン間の連続的な相互作用により厳密に制御され、多段階のカスケード反応により誘導される。これが制御不能となると重度の組織病変や臓器機能不全を引き起こす 。フザプラジブ（FZP）およびポリ硫酸ペントサン（PPS）は、炎症性疾患の治療薬として獣医療において臨床的に使用されている 。しかしながら、特に犬においては好中球接着分子およびケモカイン受容体に対するこれら薬剤の包括的な調節作用は十分に解明されていない 。本論文では、犬の内皮細胞および好中球を用いた *in vitro* 炎症モデルを用いて、FZP および PPS の効果を分子生物学的および機能的に調査した 。定量的リアルタイム PCR（qPCR）、enzyme-linked immunosorbent assay（ELISA）法、フローサイトメトリー、および静的接着アッセイを用いて、接着分子、ケモカイン、および機能的接着能を定量した 。

第 I 章では FZP に焦点を当てた結果、その抗炎症作用は、これまでに報告されている LFA-1 阻害剤としての役割を超えて、接着の初期段階を調節することが明らかになった 。検討した最高濃度（100 μM）において、内皮細胞における P-セレクチンの mRNA 発現が有意に制御された。さらに、好中球の L-セレクチンおよび PSGL-1 mRNA 発現においても、最高用量で有意な減少が観察された 。静的接着アッセイにおいて有意な阻害が認められなかったことは、FZP が LFA-1/ICAM-1 を介した強固な接着段階ではなく、主にテザリングおよびローリング段階を標的とする可能性が高いという仮説を裏付けた 。

第 II 章では、ヘパリン様化合物である PPS が、非常に選択的で内皮細胞に特化した性質を持ち、さらに細胞毒性を持たないことを示した 。本研究では、PPS は検討した濃度において内皮細胞において P-セレクチン mRNA 発現を一貫して減少させ、これは 1 μg/ml という低濃度においても認められた。一方で、ICAM-1、E-セレクチン、CXCL1、あるいは検討したいずれの好中球接着分子に対しても PPS は調節作用を示さ

なかった。機能的アッセイでも同様に阻害作用は認められず、PPS の効果はセレクチンを介したローリング段階に限定されることが示された。

検討した 2 剤を比較分析したところ、明確な相違点が明らかになった。すなわち、PPS は内皮細胞に対して選択的かつ強力な安定化剤として機能したのに対し、FZP は内皮と好中球の両方の接着経路に対して、濃度依存的に制御することがわかった。本論文は、FZP と PPS の炎症抑制効果に対する作用機序に関する重要な知見を示した。